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(57) Abstract

The *CONSTANS (CO)* gene of *Arabidopsis thaliana* and homologues from *Brassica napus* are provided and are useful for influencing flowering characteristics in transgenic plants, especially the timing of flowering.

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GENETIC CONTROL OF FLOWERING

This invention relates to the genetic control of flowering in plants and the cloning and expression of genes involved therein. More particularly, the invention relates to the cloning and expression of the *CONSTANS* (*CO*) gene of *Arabidopsis thaliana*, and homologues from other species, including *Brassica napus* and manipulation and use of the gene in plants.

Efficient flowering in plants is important, particularly when the intended product is the flower or the seed produced therefrom. One aspect of this is the timing of flowering: advancing or retarding the onset of flowering can be useful to farmers and seed producers. An understanding of the genetic mechanisms which influence flowering provides a means for altering the flowering characteristics of the target plant. Species for which flowering is important to crop production are numerous, essentially all crops which are grown from seed, with important examples being the cereals, rice and maize, probably the most agronomically important in warmer climatic zones, and wheat, barley, oats and rye in more temperate climates. Important seed products are oil seed rape and canola, sugar beet, maize, sunflower, soyabean and sorghum. Many crops which are harvested for their roots are, of course, grown annually from seed and the production of seed of any kind is very dependent upon the ability of the plant to flower, to be pollinated and to set seed. In horticulture, control of

the timing of flowering is important. Horticultural plants whose flowering may be controlled include lettuce, endive and vegetable brassicas including cabbage, broccoli and cauliflower, and carnations and geraniums.

Arabidopsis thaliana is a facultative long day plant, flowering early under long days and late under short days. Because it has a small, well-characterized genome, is relatively easily transformed and regenerated and has a rapid growing cycle, *Arabidopsis* is an ideal model plant in which to study flowering and its control.

We have discovered that one of the genes required for this response to photoperiod is the *CONSTANS* or *CO* gene, also called *FG*. We have found that plants carrying mutations of this gene flower later than their wild-types under long days but at the same time under short days, and we conclude, therefore, that the *CO* gene product is involved in the promotion of flowering under long days.

Putterill et al, *Mol. Gen. Genet.* 239: 145-157 (1993) describes preliminary cloning work which involved chromosome walking with yeast artificial chromosome (YAC) libraries and isolation of 1700kb of contiguous DNA on chromosome 5 of *Arabidopsis*, including a 300kb region containing the gene *CO*. That work fell short of cloning and identification of the *CO* gene.

We have now cloned and sequenced the *CO* gene (Putterill et al., 1995), which is provided herein.

Unexpected difficulties and complications were encountered which made the cloning harder than anticipated, as is discussed below in the experimental section.

According to a first aspect of the present invention there is provided a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide with CO function. Those skilled in the art will appreciate that "CO function" may be used to refer to the ability to influence the timing of flowering phenotypically like the *CO* gene of *Arabidopsis thaliana* (the timing being substantially unaffected by vernalisation), especially the ability to complement a *co* mutation in *Arabidopsis thaliana*, or the *co* phenotype in another species. *CO* mutants exhibit delayed flowering under long days, the timing of flowering being substantially unaffected by vernalisation (see, for example, Korneef et al. (1991)).

Nucleic acid according to the present invention may have the sequence of a *CO* gene of *Arabidopsis thaliana*, or be a mutant, derivative or allele of the sequence provided. Preferred mutants, derivatives and alleles are those which encode a protein which retains a functional characteristic of the protein encoded by the wild-type gene, especially the ability to promote flowering as discussed herein. Other preferred mutants, derivatives and alleles encode a protein which delays flowering compared to wild-type or a gene with the

sequence provided. Changes to a sequence, to produce a mutant or derivative, may be by one or more of addition, insertion, deletion or substitution of one or more nucleotides in the nucleic acid, leading to the addition, insertion, deletion or substitution of one or more amino acids in the encoded polypeptide. Of course, changes to the nucleic acid which make no difference to the encoded amino acid sequence are included.

A preferred nucleic acid sequence for a *CO* gene is shown in Figure 1, along with the encoded amino acid sequence of a polypeptide which has CO function.

The present invention also provides a vector which comprises nucleic acid with any one of the provided sequences, preferably a vector from which polypeptide encoded by the nucleic acid sequence can be expressed. The vector is preferably suitable for transformation into a plant cell. The invention further encompasses a host cell transformed with such a vector, especially a plant cell. Thus, a host cell, such as a plant cell, comprising nucleic acid according to the present invention is provided. Within the cell, the nucleic acid may be incorporated within the chromosome. There may be more than one heterologous nucleotide sequence per haploid genome. This, for example, enables increased expression of the gene product compared with endogenous levels, as discussed below.

A vector comprising nucleic acid according to the present invention need not include a promoter or other

regulatory sequence, particularly if the vector is to be used to introduce the nucleic acid into cells for recombination into the genome.

Nucleic acid molecules and vectors according to the present invention may be provided isolated from their natural environment, in substantially pure or homogeneous form, or free or substantially free of nucleic acid or genes of the species of interest or origin other than the sequence encoding a polypeptide able to influence flowering, eg in *Arabidopsis thaliana* nucleic acid other than the *CO* sequence.

Nucleic acid may of course be double- or single-stranded, cDNA or genomic DNA, RNA, wholly or partially synthetic, as appropriate.

The present invention also encompasses the expression product of any of the nucleic acid sequences disclosed and methods of making the expression product by expression from encoding nucleic acid therefor under suitable conditions in suitable host cells. Those skilled in the art are well able to construct vectors and design protocols for expression and recovery of products of recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd

edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press. Transformation procedures depend on the host used, but are well known.

The present invention further encompasses a plant comprising a plant cell comprising nucleic acid according to the present invention, and selfed or hybrid progeny and any descendant of such a plant, also any part or propagule of such a plant, progeny or descendant, including seed.

A further aspect of the present invention provides a method of identifying and cloning CO homologues from plant species other than *Arabidopsis thaliana* which method employs a nucleotide sequence derived from that shown in Figure 1. The genes whose sequences are shown in Figure 5 and Figure 6 were cloned in this way. Sequences derived from these may themselves be used in identifying and in cloning other sequences. The nucleotide sequence information provided herein, or any part thereof, may be used in a data-base search to find homologous sequences, expression products of which can be tested for ability to influence a flowering characteristic. These may have "CO function" or the ability to complement a mutant phenotype, which phenotype is delayed flowering (especially under long days), preferably the timing of flowering being substantially unaffected by vernalisation, as disclosed herein. Alternatively, nucleic acid libraries may be screened using techniques well known to those skilled in

the art and homologous sequences thereby identified then tested.

The present invention also extends to nucleic acid encoding a CO homologue obtained using a nucleotide sequence derived from that shown in Figure 1. CO homologue sequences are shown in Figures 5 and 6. Also encompassed by the invention is nucleic acid encoding a CO homologue obtained using a nucleotide sequence derived from a sequence shown in Figure 5 or Figure 6.

The *CO* protein contains an arrangement of cysteines at the amino end of the protein that is characteristic of zinc fingers, such as those contained within the GATA transcription factors (discussed by Ramain et al, 1993; Sánchez-Garciá and Rabbitts, 1994). Seven independently isolated *co* mutants have been described, and we have identified the sequence changes causing a reduction in *CO* activity in all seven cases. Five of them have alterations within regions proposed from their sequence to form zinc fingers, and the other two have changes in adjacent amino acids at the carboxy terminus of the protein. The positions of these alterations support our interpretation that *CO* encodes a protein containing zinc fingers that probably binds DNA and acts as a transcription factor.

The provision of sequence information for the *CO* gene of *Arabidopsis thaliana* enables the obtention of homologous sequences from other plant species. In Southern hybridization experiments a probe containing

the *CO* gene of *Arabidopsis thaliana* hybridises to DNA extracted from *Brassica nigra*, *Brassica napus* and *Brassica oleraceae*. Different varieties of these species display restriction fragment length polymorphisms when their DNA is cleaved with a restriction enzyme and hybridised to a *CO* probe. These RFLPs may then be used to map the *CO* gene relative to other RFLPs of known position. In this way for example, three *CO* gene homologues were mapped to linkage groups N5, N2 and N12 of *Brassica napus* (D. Lydiate, unpublished). The populations used for RFLP mapping had previously been scored for flowering time and it was demonstrated that particular alleles of the *CO* homologues segregated together with allelic variations affecting flowering time. The loci mapped to linkage groups N2 and N12 showed the most extreme allelic variation for flowering time.

Successful cloning of two *Brassica napus* homologues is described in Example 5.

This confirms that genes homologous to the *CO* gene of *Arabidopsis* regulate flowering time in other plant species.

Thus, included within the scope of the present invention are nucleic acid molecules which encode amino acid sequences which are homologues of *CO* of *Arabidopsis thaliana*. Homology may be at the nucleotide sequence and/or amino acid sequence level. Preferably, the nucleic acid sequence shares homology with the sequence

encoded by the nucleotide sequence of Figure 1, preferably at least about 50%, or 60%, or 70%, or 80% homology, most preferably at least 90% homology, from species other than *Arabidopsis thaliana* and the encoded polypeptide shares a phenotype with the *Arabidopsis thaliana* *CO* gene, preferably the ability to influence timing of flowering. These may promote or delay flowering compared with *Arabidopsis thaliana* *CO* and mutants, derivatives or alleles may promote or delay flowering compared with wild-type.

CO gene homologues may also be identified from economically important monocotyledonous crop plants such as rice and maize . Although genes encoding the same protein in monocotyledonous and dicotyledonous plants show relatively little homology at the nucleotide level, amino acid sequences are conserved. In public sequence databases we recently identified several *Arabidopsis* cDNA clone sequences that were obtained in random sequencing programmes and share homology with *CO* in regions of the protein that are known to be important for its activity. Similarly, among randomly sequenced rice cDNAs we identified one clone that shared relatively little homology to *CO* at the DNA level but showed high homology at the amino acid level. This clone, and another one that we have identified from maize, may be used to to identify the whole *CO* gene family from rice and other cereals. By sequencing each of these clones, studying their expression patterns and

examining the effect of altering their expression, genes carrying out a similar function to *CO* in regulating flowering time are obtainable. Of course, mutants, derivatives and alleles of these sequences are included within the scope of the present invention in the same terms as discussed above for the *Arabidopsis thaliana* CO gene.

Nucleic acid according to the invention may comprise a nucleotide sequence encoding a polypeptide able to complement a mutant phenotype which is delayed flowering, the timing of flowering being substantially unaffected by vernalisation. The delayed flowering may be under long days. Also the present invention provides nucleic acid comprising a nucleotide sequence which is a mutant or derivative of a wild-type gene encoding a polypeptide with ability to influence the timing of flowering, the mutant or derivative phenotype being early or delayed flowering with the timing of flowering being substantially unaffected by vernalisation. These are distinguished from the *LD* gene reported by Lee et al.

Vernalisation is low-temperature (usually just above 0°C) treatment of plant (seedlings) or seed for a period of usually a few weeks, probably about 30 days. It is a treatment required by some plant species before they will break bud or flower, simulating the effect of winter cold.

Also according to the invention there is provided a

plant cell having incorporated into its genome a sequence of nucleotides as provided by the present invention, under operative control of a regulatory sequence for control of expression. A further aspect of the present invention provides a method of making such a plant cell involving introduction of a vector comprising the sequence of nucleotides into a plant cell and causing or allowing recombination between the vector and the plant cell genome to introduce the sequence of nucleotides into the genome.

Plants which comprise a plant cell according to the invention are also provided, along with any part or propagule thereof, seed, selfed or hybrid progeny and descendants.

The invention further provides a method of influencing the flowering characteristics of a plant comprising expression of a heterologous *CO* gene sequence (or mutant, allele, derivative or homologue thereof, as discussed) within cells of the plant. The term "heterologous" indicates that the gene/sequence of nucleotides in question have been introduced into said cells of the plant using genetic engineering, ie by human intervention. The gene may be on an extra-genomic vector or incorporated, preferably stably, into the genome. The heterologous gene may replace an endogenous equivalent gene, ie one which normally performs the same or a similar function in control of flowering, or the inserted sequence may be additional to the endogenous

gene. An advantage of introduction of a heterologous gene is the ability to place expression of the gene under the control of a promoter of choice, in order to be able to influence gene expression, and therefore flowering, according to preference. Furthermore, mutants and derivatives of the wild-type gene, eg with higher or lower activity than wild-type, may be used in place of the endogenous gene.

The principal flowering characteristic which may be altered using the present invention is the timing of flowering. Under-expression of the gene product of the *CO* gene leads to delayed flowering (as suggested by the *co* mutant phenotype); over-expression may lead to precocious flowering (as demonstrated with transgenic *Arabidopsis* plants carrying extra copies of the *CO* gene and by expression from CaMV 35S promoter). This degree of control is useful to ensure synchronous flowering of male and female parent lines in hybrid production, for example. Another use is to advance or retard the flowering in accordance with the dictates of the climate so as to extend or reduce the growing season. This may involve use of anti-sense or sense regulation.

A second flowering characteristic that may be altered is the distribution of flowers on the shoot. In *Arabidopsis*, flowers develop on the sides but not at the apex of the shoot. This is determined by the location of expression of the *LEAFY* genes (Weigel et al., 1992), and mutations such as *terminal flower* (Shannon and

Meeks-Wagner, 1991) that cause *LEAFY* to be expressed in the apex of the shoot also lead to flowers developing at the apex. There is evidence that *CO* is required for full activity of *LEAFY* (Putterill et al., 1995), and therefore by increasing or altering the pattern of *CO* expression the level and positions of *LEAFY* expression, and therefore of flower development, may also be altered. This is exemplified in Example 4. This may be employed advantageously in creating new varieties of horticultural species with altered arrangements of flowers.

The nucleic acid according to the invention, such as a *CO* gene or homologue, may be placed under the control of an externally inducible gene promoter to place the timing of flowering under the control of the user. The use of an inducible promoter is described below. This is advantageous in that flower production, and subsequent events such as seed set, may be timed to meet market demands, for example, in cut flowers or decorative flowering pot plants. Delaying flowering in pot plants is advantageous to lengthen the period available for transport of the product from the producer to the point of sale and lengthening of the flowering period is an obvious advantage to the purchaser.

The term "inducible" as applied to a promoter is well understood by those skilled in the art. In essence, expression under the control of an inducible promoter is "switched on" or increased in response to an

applied stimulus. The nature of the stimulus varies between promoters. Some inducible promoters cause little or undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Other inducible promoters cause detectable constitutive expression in the absence of the stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct stimulus. The preferable situation is where the level of expression increases upon application of the relevant stimulus by an amount effective to alter a phenotypic characteristic. Thus an inducible (or "switchable") promoter may be used which causes a basic level of expression in the absence of the stimulus which level is too low to bring about a desired phenotype (and may in fact be zero). Upon application of the stimulus, expression is increased (or switched on) to a level which brings about the desired phenotype.

Suitable promoters include the Cauliflower Mosaic Virus 35S (CaMV 35S) gene promoter that is expressed at a high level in virtually all plant tissues (Benfey et al, 1990a and 1990b); the maize glutathione-S-transferase isoform II (GST-II-27) gene promoter which is activated in response to application of exogenous safener (WO93/01294, ICI Ltd); the cauliflower meri 5 promoter that is expressed in the vegetative apical meristem as well as several well localised positions in the plant body, eg inner phloem, flower primordia,

branching points in root and shoot (Medford, 1992; Medford et al, 1991) and the *Arabidopsis thaliana LEAFY* promoter that is expressed very early in flower development (Weigel et al, 1992).

When introducing a chosen gene construct into a cell, certain considerations must be taken into account, well known to those skilled in the art. The nucleic acid to be inserted should be assembled within a construct which contains effective regulatory elements which will drive transcription. There must be available a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into the endogenous chromosomal material either will or will not occur. Finally, as far as plants are concerned the target cell type must be such that cells can be regenerated into whole plants.

Plants transformed with a DNA segment containing the sequence may be produced by standard techniques for the genetic manipulation of plants. DNA can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by Agrobacterium exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966), electroporation (EP 290395, WO 8706614) or other forms of direct DNA uptake (DE 4005152, WO 9012096, US

4684611). Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous species. Although Agrobacterium has been reported to be able to transform foreign DNA into some monocotyledonous species (WO 92/14828), microprojectile bombardment, electroporation and direct DNA uptake are preferred where Agrobacterium is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, eg bombardment with Agrobacterium coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with Agrobacterium (EP-A-486233).

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention.

In the present invention, over-expression may be achieved by introduction of the nucleotide sequence in a sense orientation. Thus, the present invention provides a method of influencing a flowering characteristic of a plant, the method comprising causing or allowing expression of the polypeptide encoded by the nucleotide

sequence of nucleic acid according to the invention from that nucleic acid within cells of the plant. (See Example 4.)

Under-expression of the gene product polypeptide may be achieved using anti-sense technology or "sense regulation". The use of anti-sense genes or partial gene sequences to down-regulate gene expression is now well-established. DNA is placed under the control of a promoter such that transcription of the "anti-sense" strand of the DNA yields RNA which is complementary to normal mRNA transcribed from the "sense" strand of the target gene. For double-stranded DNA this is achieved by placing a coding sequence or a fragment thereof in a "reverse orientation" under the control of a promoter. The complementary anti-sense RNA sequence is thought then to bind with mRNA to form a duplex, inhibiting translation of the endogenous mRNA from the target gene into protein. Whether or not this is the actual mode of action is still uncertain. However, it is established fact that the technique works. See, for example, Rothstein et al, 1987; Smith et al, 1988; Zhang et al, 1992.

Thus, the present invention also provides a method of influencing a flowering characteristic of a plant, the method comprising causing or allowing anti-sense transcription from nucleic acid according to the invention within cells of the plant.

When additional copies of the target gene are

inserted in sense, that is the same, orientation as the target gene, a range of phenotypes is produced which includes individuals where over-expression occurs and some where under-expression of protein from the target gene occurs. When the inserted gene is only part of the endogenous gene the number of under-expressing individuals in the transgenic population increases. The mechanism by which sense regulation occurs, particularly down-regulation, is not well-understood. However, this technique is also well-reported in scientific and patent literature and is used routinely for gene control. See, for example, van der Krol, 1990; Napoli et al, 1990; Zhang et al, 1992.

Thus, the present invention also provides a method of influencing a flowering characeristic of a plant, the method comprising causing or allowing expression from nucleic acid according to the invention within cells of the plant. This may be used to suppress activity of a polypeptide with ability to influence a flowering characteristic. Here the activity of the polypeptide is preferably suppressed as a result of under-expression within the plant cells.

As stated above, the expression pattern of the *CO* gene may be altered by fusing it to a foreign promoter. For example, International patent application WO93/01294 of Imperial Chemical Industries Limited describes a chemically inducible gene promoter sequence isolated from a 27 kD subunit of the maize glutathione-S-

transferase, isoform II gene (GST-II-27) (see Figure 2). It has been found that when linked to an exogenous gene and introduced into a plant by transformation, the GST-II-27 promoter provides a means for the external regulation of the expression of that exogenous gene. The structural region of the *CO* gene is fused to the GST-II-27 promoter downstream of the translation start point shown in Figure 2.

The GST-II-27 gene promoter has been shown to be induced by certain chemical compounds which can be applied to growing plants. The promoter is functional in both monocotyledons and dicotyledons. It can therefore be used to control gene expression in a variety of genetically modified plants, including field crops such as canola, sunflower, tobacco, sugarbeet, cotton; cereals such as wheat, barley, rice, maize, sorghum; fruit such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, and melons; and vegetables such as carrot, lettuce, cabbage and onion. The GST-II-27 promoter is also suitable for use in a variety of tissues, including roots, leaves, stems and reproductive tissues.

Accordingly, the present invention provides in a further aspect a gene construct comprising an inducible promoter operatively linked to a nucleotide sequence provided by the present invention, such as the *CO* gene of *Arabidopsis thaliana*, a homologue from another plant species or any mutant, derivative or allele thereof.

This enables control of expression of the gene. The invention also provides plants transformed with said gene construct and methods comprising introduction of such a construct into a plant cell and/or induction of expression of a construct within a plant cell, by application of a suitable stimulus, an effective exogenous inducer. The promoter may be the GST-II-27 gene promoter or any other inducible plant promoter.

Promotion of *CO* activity to cause early flowering

Mutations that reduce *CO* activity cause late flowering under inductive long day conditions, indicating *CO* involvement in promoting flowering under long days. It is probably not required under non-inductive short days because *co* mutations have no effect on flowering time under these conditions. The *CO* transcript is present at very low abundance under long days and has only been detected by using PCR to amplify cDNA. The observation that some transgenic plants harbouring a T-DNA containing *CO* flowered slightly earlier than wild type under long days and considerably earlier than wild type under short days, suggests that, particularly under non-inductive short days, the level of the *CO* transcript is limiting on flowering time. This suggests that flowering could be manipulated by using foreign promoters to alter the expression of the gene:

Causing early flowering under non-inductive conditions

Manipulation of *CO* transcript levels under non-inductive conditions may lead to early, or regulated, flowering. Promoter fusions such as those disclosed herein enable expression of *CO* mRNA at a higher level than that found in wild-type plants under non-inductive conditions. Use of CaMV35S or meri 5 fusions leads to early flowering while use of GSTII fusions leads to regulated flowering.

Causing early flowering under inductive conditions

Wild-type *Arabidopsis* plants flower extremely quickly under inductive conditions and the *CO* gene is expressed prior to flowering, although at a low level. Nevertheless, some transgenic wild-type plants containing extra copies of *CO* have been shown to flower slightly earlier than wild-type plants. The level of the *CO* product may be increased by introduction of promoter, eg CaMV35S or meri 5, fusions. Inducible promoters, such as GSTII, may be used to regulate flowering, eg by first creating a *CO* mutant of a particular species and then introducing an inducible promoter-*CO* fusion capable of complementation of the mutation in a regulated fashion.

Inhibition of *CO* activity to cause late flowering

co mutations cause late flowering of *Arabidopsis*. Transgenic approaches may be used to reduce *CO* activity and thereby delay or prevent flowering in a range of plant species. A variety of strategies may be employed.

Expression of sense or anti-sense RNAs

In several cases the activity of endogenous plant genes has been reduced by the expression of homologous antisense RNA from a transgene, as discussed above. Similarly, the expression of sense transcripts from a transgene may reduce the activity of the corresponding endogenous copy of the gene, as discussed above. Expression of a *CO* antisense or sense RNA should reduce activity of the endogenous gene and cause late flowering.

Expression of modified versions of the *CO* protein

Transcription factors and other DNA binding proteins often have a modular structure in which amino acid sequences required for DNA binding, dimerisation or transcriptional activation are encoded by separate domains of the protein (Reviewed by Ptashne and Gann, 1990). This permits the construction of truncated or fusion proteins that display only one of the functions of the DNA binding protein. In the case of *CO*, modification of the gene *in vitro* and expression of modified versions of the protein may lead to dominant inhibition of the endogenous, intact protein and thereby delay flowering. This may be accomplished in various ways, including the following:

Expression of a truncated *CO* protein encoding only the DNA binding region.

The zinc-finger containing region of *CO* may be required and sufficient to permit binding to DNA. If a truncated or mutated protein that only encodes the DNA

binding region were expressed at a higher level than the endogenous protein, then most of the *CO* binding sites should be occupied by the mutated version thereby preventing binding of the fully active endogenous protein. Binding of the mutant protein would have the effect of preventing *CO* action, because the mutated protein would not contain any other regions of *CO* that might be involved in biological processes such as transcriptional activation, transcriptional inhibition or protein-protein interaction.

In vitro analysis of a murine transcription factor GF1 that contains zinc-fingers similar to those of *CO*, suggests that a truncated *CO* protein with the properties described above could be designed. Martin and Orkin (1990) demonstrated that a truncated version of GF1 containing only the zinc fingers retained DNA binding activity, but was incapable of transcriptional activation. Similarly, the zinc-finger containing *PANNIER* protein of *Drosophila melanogaster* is required to repress activation of genes required for bristle formation. Mutations in a domain that does not contain the zinc fingers caused dominant super-repression of gene activity, probably because these proteins bind DNA but no not interact with other proteins in the way that the wild-type protein does (Ramain et al, 1993).

Expression of a mutant *CO* protein not encoding the DNA binding domain

A second form of inhibitory molecule may be

designed if *CO* must dimerise, or form complexes with other proteins, to have its biological effect, and if these complexes can form without a requirement for *CO* being bound to DNA. In this case expression of a *CO* protein that is mutated within the DNA-binding domain, but contains all of the other properties of the wild-type protein, would have an inhibitory effect. If the mutant protein were present at a higher concentration than the endogenous protein and *CO* normally forms dimers, then most of the endogenous protein would form dimers with the mutant protein and would not bind DNA. Similarly, if *CO* forms complexes with other proteins, then the mutant form of *CO* would participate in the majority of these complexes which would then not bind DNA.

Mutant forms of DNA-binding proteins with these properties have been reported previously. For example, in yeast cells expression of a protein containing the transcriptional activation domain of GAL4 was able to reduce the expression of the *CYC1* gene. *CYC1* is not normally activated by GAL4, so it was proposed that the GAL4 activating domain sequesters proteins required for *CYC1* activation (GIll and Ptashne, 1988). Similarly, mutations in the zinc finger region of the *PANNIER* protein of *Drosophila melanogaster* have a dominant phenotype, probably because the mutant proteins sequester proteins essential for *PANNIER* activity and reduce their availability to interact with wild-type

protein (Ramain, 1993).

Aspects and embodiments of the present invention will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

In the Figures:

Figure 1 shows a nucleotide sequence according to one embodiment of the invention, being the sequence of the *CO* ORF obtained from *Arabidopsis thaliana* (SEQ ID NO. 1), and the predicted amino acid sequence (SEQ ID NO. 2). The nucleotide sequence is shown above the amino acid sequence. The region shown in bold is thought to encompass both zinc finger domains.

Figure 2 shows the nucleotide sequence of the GST-II-27 gene promoter (SEQ ID NO. 3). The fragment used to make the fusion was flanked by the *HindIII* and *NdeI* sites that are shown in bold.

Figure 3 shows the nucleotide sequence of the genomic DNA comprising the *CO* gene obtained from *Arabidopsis thaliana*, including the single intron, promoter sequences and sequences present after the translational termination codon (SEQ ID NO. 4). The genomic region shown starts 2674 bp upstream of the translational start site, and ends just after the polyadenylation site. The *CO* open reading frame is shown in bold, and is interrupted by the single intron.

Figure 4 shows the pJIT62 plasmid used as a source of the CaMV 35S promoter. The *KpnI-HindIII* fragment, shown as a dark coloured thick line, was used as a source of the promoter.

Figure 5 shows a nucleotide sequence according to a further embodiment of the invention, being a *CO* ORF obtained from *Brassica napus* (SEQ ID NO. 5), and the predicted amino acid sequence (SEQ ID NO. 6).

Figure 6 shows a nucleotide sequence according to a further embodiment of the invention, being a second *CO* ORF obtained from *Brassica napus* (SEQ ID NO. 7), and the predicted amino acid sequence (SEQ ID NO. 8).

EXAMPLE 1 - cloning and analysis of a CO gene

Cosmid and RFLP markers.

DNA of λ CHS2 was obtained from R. Feinbaum (Massachusetts General Hospital (MGH), Boston). Total DNA was used as radiolabelled probe to YAC library colony filters and plant genomic DNA blots. Cosmids g6833, 17085, 17861, 19027, 16431, 14534, g5962 and g4568 were obtained from Brian Hauge (MGH, Boston), cultured in the presence of 30 μg/ml kanamycin, and maintained as glycerol stocks at - 70°C. Total cosmid DNA was used as radiolabelled probe to YAC library colony filters and plant genomic DNA blots. Cosmid pCIT1243 was provided by Elliot Meyerowitz (Caltech, Pasadena), cultured in the presence of 100 μg/ml streptomycin/spectinomycin and maintained as a glycerol

stock at - 70° C. pCIT30 vector sequences share homology to pYAC4 derived vectors, and therefore YAC library colony filters were hybridised with insert DNA extracted from the cosmid. Total DNA of pCIT1243 was used as radiolabelled probe to plant genomic DNA blots.

YAC libraries.

The EG, abi and S libraries were obtained from Chris Somerville (Michigan State University). The EW library was obtained from Jeff Dangl (Max Delbrück Laboratory, Cologne) and the Yup library from Joe Ecker (University of Pennsylvania). Master copies of the libraries were stored at -70°C (as described by Schmidt et al. Aust. J. Plant Physiol. 19: 341-351 (1992)). The working stocks were maintained on selective Kiwibrew agar at 4°C. Kiwibrew is a selective, complete minimal medium minus uracil, and containing 11% Casamino acids. Working stocks of the libraries were replated using a 96-prong replicator every 3 months.

Yeast colony filters.

Hybond-N (Amersham) filters (8cm x 11cm) containing arrays of yeast colony DNA from 8-24 library plates were produced and processed (as described by Coulson et al. Nature 335:184-186 (1988) and modified (as described by Schmidt and Dean Genome Analysis, vol.4: 71-98 (1992)). Hybridisation and washing conditions were according to the manufacturer's instructions. Radiolabelled probe

DNA was prepared by random-hexamer labelling.

Yeast chromosome preparation and fractionation by pulsed field gel electrophoresis (PFGE).

Five millilitres of Kiwibrew was inoculated with a single yeast colony and cultured at 30°C for 24 h. Yeast spheroplasts were generated by incubation with 2.5mg/ml Novozym (Novo Biolabs) for 1 h at room temperature. Then 1 M sorbitol was added to bring the final volume of spheroplasts to 50 µl. Eighty microlitres of molten LMP agarose (1% InCert agarose, FMC) in 1 M sorbitol was added to the spheroplasts, the mixture was vortexed briefly and pipetted into plug moulds. Plugs were placed into 1.5ml Eppendorf tubes and then incubated in 1 ml of 1 mg/ml Proteinase K (Boehringer Mannheim) in 100 mMEDTA, pH 8, 1% Sarkosyl for 4 h at 50°C. The solution was replaced and the plugs incubated overnight. The plugs were washed three times for 30 min each with TE and twice for 30 min with 0.5 x TVBE. PFGE was carried out using the Pulsaphor system (LKB). One-third of a plug was loaded onto a 1% agarose gel and electrophoresed in 0.5 x TBE at 170 V, 20 s pulse time, for 36 h at 4°C. DNA markers were concatemers of λ DNA prepared as described by Bancroft and Wolk, Nucleic A Res. 16:7405-7418 (1988). DNA was visualised by staining with ethidium bromide.

Yeast genomic DNA for restriction enzyme digestion and

inverse polymerase chain rection (IPCR).

Yeast genomic DNA was prepared essentially as described by Heard et al. (1989) except that yeast spheroplasts were prepared as above. Finally, the DNA was extracted twice with phenol/chloroform, once with chloroform and ethanol precipitated. The yield from a 5ml culture was about 10μg DNA.

Isolation of YAC end fragments by IPCR.

Yeast genomic DNA (100 ng) was digested with *Alu*I, *Hae*III, *Eco*RV or *Hinc*II. The digestions were phenol-chloroform extracted once and then ethanol precipitated. The DNA fragments were circularised by ligation in a volume of 100 μl over-night at 16°C in the presence of 2 U ligase (BRL). After incubation of the ligation mixture at 65°C for 10 min, IPCR was carried out on 10 μl ligation mixture using inverse primer pairs. The IPCR conditions and C and D primer pairs have been described by Schmidt et al. (1992). The JP series are from M. Hirst (IMM Molecular Genetics Group, Oxford).

After digestion with the indicated enzymes, the following primer pairs were used:

For left-end IPCR:

*Alu*I, *Eco*RV; D71 5'tcctgctcgcttcgctactt3'
and C78 5'gcgatgctgtcggaatggac3'

*Hae*III; JP1 5'aagtactctcggtagccaag3'
and JP5 5'gtgtggtcgccatgatcgcg3'.

For right-end IPCR:

*Alu*I, *Hinc*II; C69 5'ctgggaagtgaatggagacata3'

and C70 5'aggagtcgcataagggagag3'

*Hae*III; C69 and JP4 5'ttcaagctctacgccgga3'.

Aliquots of the IPCR reactions were checked by electrophoresis on a 1.5% agarose gel and the 1 μl of the reaction was re-amplified by PCR using the conditions and F primer series recommended by I. Hwang (MGH, Boston). Conditions for re-amplification were the same as for IPCR, except that 30 cycles (1 min, 94°C; 1 min, 45°C; and 3 min, 72°C) were used. The F primers anneal very near the cloning site and so reduce the amount of vector sequence present in the PCR product. In addition they introduce a *FokI* site very close to the destroyed cloning site of EW and S YACs.

The primers used for re-amplification of left-end IPCR products were as follows:

For EG, abi and S YACs:

*Alu*I, F2 5'acgtcggatgctcactatagggatc3'

and C77 5'gtgataaactaccgcattaaagc3';

*Hae*III, F2 and JP5; *Eco*RV, F2 and 78.

For EW and Yup YACs: *Alu*I,

F6 5'acgtcggatgactttaatttattcacta3'

and C77; *Hae*III, F6 and JP5; *Eco*RV, F6 and C78.

The following primers were used for re-amplification of the right-end IPCR products:

For EG, abi and S YACs: *Alu*I,

F3 5'gacgtggatgctcactaaagggatc3'

and C71 5'agagccttcaacccagtcag3'; *Hae*III, F3 and JP4; *Hinc*II, F3 and C70.

For EW and YUP YACs: *Alu*I,

F7 5'acgtcggatgccgatctcaagatta3'

and C77; *Hae*III, F7 and JP4; 4HincII, F7 and C70.

The resulting PCR product was purified by cleaving with the enzyme originally used in the digestion together with *Bam*HI (EG and abi YACs) or *Eco*RI (Yup YACs) and separated on 1% LMP agarose gels. The YAC end probes were radiolabelled using random priming in molten agarose, and in appropriate cases digested with *Fok*I to remove vector sequences and then used as hybridisation probes.

Isolation of YAC left-end probes by plasmid rescue.

Plasmid rescue of YAC left-end fragments from EG, abi and EW YACs was carried out as described by Schmidt et al. (1992).

Isolation of plant genomic DNA.

Plant genomic DNA was isolated from glasshouse grown plants essentially as described by Tai and Tanksley, Plant Mol. Biol. Rep. 8: 297-303 (1991), except that the tissue was ground in liquid nitrogen and the RNase step omitted. Large-scale (2.5-5 g leaves) and miniprep (3-4 leaves) DNA was prepared using this method.

Gel blotting and hybridisation conditions.

Gel transfer to Hybond-N, hybridisation and washing conditions were according to the manufacturer's instructions, except that DNA was fixed to the filters by UV Stratalinker treatment (1200 μJ x 100; Stratagene) and/or baked at 80°C for 2 h. Radiolabelled DNA was prepared by random hexamer labelling.

RFLP analysis.

Two to three micrograms of plant genomic DNA was prepared from the parental plants used in the crosses and cleaved in a 300 μl volume with 1 of 17 restriction enzymes: *Dra*I, *Bcl*I, *Cfo*I, *Eco*RI, *Eco*RV, *Hinc*II, *Bgl*III, *Rsa*I, *Bam*HI, *Hind*III, *Sac*I, *Alu*I, *Hinf*I, *Sau*3A, *Taq*I and *Mbo*I. The digested DNA was ethanol precipitated and separated on 0.7% agarose gels and blotted onto Hybond-N filters. Radiolabelled cosmid λ or YAC end probe DNA was hybridised to the filters to identify RFLPs.

Selection of plants carrying recombination events in the vicinity of co.

The first step in selecting recombinants was to create lines carrying the *co* mutation and closely linked markers. This was done twice for different flanking markers. In the first experiment a Landsberg *erecta* line carrying the *co-2* allele (Koornneef et al. 1991) and *tt4* was made. The *tt4* mutation prevents the production of anthocyanin and has previously been

suggested to be a lesion in the gene encoding chalcone synthase, because this map to a similar location (Chang et al. 1988). The double mutant was crossed to an individual of the Niederzenz ecotype and the resulting hybrid self-fertilised to produce an F_2 population. This population was then screened phenotypically for individuals in which recombination had occurred between *co-2* and *tt4*. In addition, F_2 plants homozygous for both mutations were used to locate marker RFLP g4568 relative to *co-2*.

The second experiment was performed by using two marked lines as parents. The first of these contained *chp7* in a Landsberg *erecta* background and was derived by Maarten Koornneef (Wageningen) from a cross between a line of undefined background (obtained from George Rédei) to Landsberg *erecta*. The second parent contained markers *lu* and *alb2*. This was selected by Maarten Koornneef from a cross of a plant of S96 background carrying the *alb2* mutation (M4-6-18; Relichová 1976) to a line containing *co-1* and *lu* (obtained by Koornneef from J. Relichová, but originally from Cr. Rédei). The *chp7*, *co-1* line was then crossed to the *lu*, *alb2* line and an F_2 population derived by self-fertilisation of the hybrid. This population was used to isolate the recombinants with crossovers between *lu* and *co-1* and between *co-1* and *alb2*. Both classes of recombinants were recognised phenotypically as *lu* homozygotes. These are only present if recombination occurs between *lu* and

alb2, because *alb2* is lethal when homozygous.

Isolation of the *CO* (FG) locus:

The *CO* gene is located on the upper arm of chromosome 5 and is 2cM proximal to *tt4*. The average physical distance in 1cM in Arabidopsis is approximately 140 kb. The distance from CHS to *CO* might be expected therefore to be ca. 300 kb.

We started by hybridising 4 RFLP markers that are closely linked (within ca. 2cM) to CHS to the EG and EW YAC libraries. This produced 18 hybridising YACs. These were run out on pulse field gels, Southern blotted and hybridised to the appropriate RFLP clone. This confirmed the colony hybridisation result and measured the size of the YACs; they ranged from 50 kb to 240 kb in size. The YACs were then digested with restriction enzymes, hybridised to RFLP marker DNA and the pattern of fragments compared to that of the marker. This allowed us to determine whether they contained all the fragments in the RFLP marker or only some of them and permitted us to deduce how the YACs lay in relation to each other. In most cases this arrangement was later confirmed by the isolation of inverse polymerise chain reaction (PCR) generated fragments which are located at the ends of the *Arabidopsis* DNA inserted within the YAC, and hybridisation of these to the appropriate overlapping YACs.

The short contigs around the RFLP markers were than

extended. We obtained two sets of overlapping cosmid clones from this area and used the appropriate ones against the YAC libraries. This identified two new YACs. End probes derived from most of the 20 YACs we had identified were then used to screen the libraries and new YACs extending the cloned region in both directions were identified. In all a detailed analysis of 67 YACs was necessary. It allowed us to assemble one contiguous segment of *Arabidopsis* DNA which includes RFLP markers 6833, CHS, pCIT1243 and 5962 and is approximately 1700 kb long.

The location of *CO* within the contig was determined by detailed RFLP analysis after the isolation of recombinants containing cross-overs very closely linked to *CO*. The recombinants were identified by using flanking phenotypic markers. First we made a Landsberg *erecta* chromosome marked with *co* and *tt4*. Then we crossed this to Niedersenz and screened 1200 F2 plants for recombinant chromosomes carrying cross-overs between *co* and *tt4*. In this way we found twelve recombinants which were confirmed by scoring the phenotypes of their progeny. The rarity of these recombinants confirmed the extremely close linkage between *tt4* and *co*. These recombinants were then used to locate *CO* on the contig. For example, some of them contain Landsberg DNA on the *tt4* side of the cross-over and Niedersenz DNA on the *co* side. DNA isolated during our walk was positioned relative to *CO* by using small fragments as RFLP markers

and hybridising them to the DNA extracted from the recombinants. We used a similar approach on the proximal side by screening for recombinants between *co* and *alb2*. This work initially located *CO* between two YAC end probes which are approximately 300 kb apart.

To locate *CO* more accurately within the 300 kb, more cross overs between *co* and the flanking phenotypic markers were screened for. Using a similar rationale as that described earlier, a total of 46 cross-overs between *co* and *alb2* (an interval of 1.6cM proximal to *CO*), and 135 between *co* and *lu* (an interval of 5.3cM distal to *CO*) were identified and analysed with appropriate RFLP markers derived from our contig. This located the gene to a very short region defined by two YAC end probes. These were used to screen a cosmid library provided to us by University of Minnesotta, and a short cosmid contig containing 3 cosmids that spanned the entire region was constructed. Analysis of these cosmids indicated that the detailed RFLP mapping had located *CO* to a region approximately 38 kb long.

To position the gene within the cosmids, each of them was introduced into *co* mutants and the resulting plants examined to determine which of the cosmids corrected the *co* mutant phenotype. Roots of plants homozygous for *co-2* and *tt4* mutations were co-cultivated with *Agrobacterium* strains containing each cosmid (Olszewski and Ausubel, 1988; Valvekens et al 1989) and kanamycin resistant plants regenerated. The regenerants

(T1 generation) were self-fertilised and their progeny sown on medium containing kanamycin to confirm that they contained the T-DNA (Table 1).

A total of 5 independent transformants containing cosmid A, 9 containing cosmid B and 13 containing cosmid C produced kanamycin resistant T2 progeny and were studied further. The flowering time of 20-40 plants from each of these T2 families was measured in the long day greenhouse. All of the progeny of transgenic plants made with cosmid A flowered as late as the *co-2* mutants, suggesting that this cosmid did not contain the *CO* gene. However, several of the families derived from plants containing cosmids B and C included early flowering individuals. In total, 6 of the 9 families derived from plants harboring cosmid B and 12 of the 13 derived from those carrying cosmid C contained plants that flowered as early as wild-type. All of these early-flowering individuals produced light coloured seeds indicating that they carried the *tt4* mutation present in the line used for the transformation, and therefore were not simply the result of the experiment being contaminated with seeds of wild-type plants (Experimental Procedures). These results strongly suggest that the *CO* gene is contained in both cosmids B and C.

Further experiments were carried out in the T3 generation to confirm the complementation results. A total of five T2 early-flowering plants derived from cosmid B and six from cosmid C were self fertilised and

studied further in the T3 generation. Each of the T2 plants chosen for this analysis was derived from a different transformant, was the earliest flowering plant in the T2 family and was a member of a family that had shown a ratio of 3 kanamycin resistant seedlings for each kanamycin sensitive, and therefore probably contained the transgene at only one locus (Table 1). All of the seedlings in these T3 families were resistant to kanamycin demonstrating that the parental T2 plants were homozygous for the T-DNA. This demonstrated that the earliest flowering T2 plants were homozygous for the *CO* transgene.

Under the long-day conditions used the *co-2* mutant plants flowered considerably later than the wild-type controls (Table 1). The T3 plants flowered at least as early as wild-type under defined long-day conditions, and some individuals flowered earlier than wild-type (Table 1). This analysis confirmed that cosmids B and C can correct the effect of the *co-2* mutation on flowering time under long days, suggesting that both of these cosmids contained *CO*, and therefore that the gene was in the region of overlap between them. This region was 6.5 kb long.

We determined the sequence of the 6.5 kb that was shared by cosmids B and C. This contains only one gene that we can readily identify from the DNA sequence. The polymerase chain reaction was used to amplify this gene from three independently isolated *co* mutants, and

sequencing of these genes demonstrated that all three contained mutations. This, together with the complementation analysis, is conclusive evidence that this is the *CO* gene. The predicted amino acid sequence of *CO* shows no homology to previously reported genes. However, the amino terminus contains two regions that are predicted to form zinc fingers, suggesting that the protein product binds to DNA and is probably a transcription factor.

Unexpected difficulties in identifying *CO* within the 300kb region defined by REG17B5 and LEW4A9

1. Locating the gene by more detailed RFLP mapping and complementation

As mentioned, Putterill et al, *Mol. Gen. Genet.* 239:145-157 (1993) described location of *CO* to within a region of 300kb. To locate *CO* more accurately by RFLP mapping, two materials were required: more recombinants carrying cross-overs within the 300kb region, and more RFLP markers to use as probes against these recombinants.

Recombinants between *lu* and *co* or between *co* and *alb2* were selected. A total of 68 cross-overs in the 1.6 cM between *lu* and *co* were identified, and 128 in the 5.3cM between *co* and *alb2*. This is equivalent to 196 cross-overs in 6.8cM, or an average of 29 cross-overs per cM. Among these recombinants, cross-overs within

the 300kb were unexpectedly under-represented: 300kb is equivalent to around 1.5cM, so 43 (29 x 1.5) cross-overs would be expecetd in this region. Only 23 were found.

The analysis of these cross-overs was also difficult because none of the YAC end probes that fell within the 300kb could be used as RFLP probes. This was due to none of them detecting RFLPs between the parental lines used to make the recombinants. One RFLP marker (pCIT1243) was available within the region, and when this was used to analyse the recombinants it was found to be between REG17B5 and *CO*, thereby positioning the gene between pCIT1243 and LEW4A9. However, a more accurate position of the gene could not be achieved by this method because of the lack of suitable probes.

The distribution of cross-overs between pCIt1243 and LEW4A9 was asymmetric: there was one between pCIT1243 and *CO* and 19 between *CO* and LEW4A9. We guessed that the gene was likely to be close to pCIT1243. A pool of probes (LEG4C9, Labi19E1, pCIT1243, LEG21H11 and REG4C9) from this region was therefore used to screen a cosmid library to provide a series of cosmid clones extending from pCIT1243 towards LEW4A9. Analysis of these clones with individual probes showed that the three cosmids A, B and C extended from pCIT1243 in the direction required. These were then used as RFLP markers and the gene demonstrated to be on the cosmids.

The procedure was therefore more complex than that envisaged in the Putterill et al paper because of the

difficulty in making enough recombinants within the 300kb region, and in identifying suitable RFLP markers.

2. Identifying the gene by complementation

The three cosmids A, B and C were introduced into mutant plants, and it was shown that B and C could correct the effect of the mutation. The gene must therefore be on the DNA shared by B and C, but the method proposed in the Putterill paper for final identification of the *CO* gene failed. It had been assumed that one would be able to identify a transcript for *CO* by using the complementing DNA as a probe against Northern blots, or that one of the seven alleles would show a re-arrangement on Southern blots that would lead to the gene. In fact, we could not detect the *CO* transcript on Northern blots nor any re-arrangment indicative of where the gene might be.

The failure of this approach led us to sequence the genomic DNA that complemented the mutation. Computer analysis of this DNA identified two open reading frames adjacent to each other and we guessed that these might represent the *CO* gene. We still had no evidence that thes ORFs were actively transcribed, as one would expect for a gene, because no transcript was detectable on Northern blots and no cDNA was detected in several cDNA libraries. We therefore used the polymerase chain reaction (PCR) to amplify a cDNA from RNA preparations. This showed that thse two ORFs did indeed represent one

active gene. Sequencing *co* alleles then confirmed that they contained single base changes, or in one case a 9bp deletion, that would not have been detected by the approaches proposed in the Putterill et al paper.

Gene Structure

To determine the gene structure, a cDNA for the *CO* gene was identified using RT-PCR (Experimental Procedures). The sequence of the cDNA contains an 1122 bp ORF that is derived from both ORFs identified in the genomic sequence by removal of a 233 bp intron. Translation of this open reading frame is predicted to form a protein containing 373 amino acids with a molecular mass of 42 kd. The transcription start site was not determined, but an in frame translation termination codon is located three codons upstream of the ATG, indicating that the entire translated region was identified. The 3' end of the transcript was located by sequencing four fragments produced by 3'-RACE. They all contained the poly-A tail at different positions within 5 bases of each other.

Available data bases were searched for proteins sharing homology with the predicted translation product of the *CO* gene. Searching the PROSITE directory detected no motifs within the *CO* protein. Moreover, a FASTA search comparing the *CO* protein sequence with those in GenBank detected no significant homologies. Direct comparison of the *CO* sequence with that of

LUMINIDEPENDENS, the other flowering time gene cloned from *Arabidopsis* (Lee et al, 1994), detected no homology. However, analysis of the protein sequence by eye identified a striking arrangement of cysteine residues that is present in two regions near the amino terminus of the *CO* protein. Each of these regions contains four cysteines in a $C\text{-}X_2\text{-}C\text{-}X_{16}\text{-}C\text{-}X_2\text{-}C$ arrangement, that is similar to the zinc-finger domains of GATA-1 transcription factors ($C\text{-}X_2\text{-}C\text{-}X_{17}\text{-}C\text{-}X_2\text{-}C$).

Comparison of two 43 amino acid stretches that are directly adjacent to each other within the predicted *CO* protein sequence and each of which contains one of the proposed zinc fingers, indicates striking homology: 46% of the amino acids are identical and 86% are either identical or related. The conservation is most apparent on the carboxy side of each finger, which is again reminiscent of GATA1 transcription factors, in which this region is a basic domain required for DNA binding and is highly conserved (Trainor et al, 1990; Brendel and Karlin, 1989; Ramain et al, 1993). In the *CO* protein this region is also positively charged: there is a net positive charge of 6 in the region adjacent to the amino finger and of 3 in the one next to the carboxy finger.

Comparison of the *CO* protein sequence of the *CO* zinc fingers with 116 amino acids that contain the zinc fingers of hGATA1 and are conserved between members of the GATA1 family (see Ramain et al, 1993) using the

FASTA programme of the Wisconsin package identified one 81 amino acid region of homology that spans both zinc fingers of *CO* and aligns the cysteines of the zinc fingers of hGATA1 and those of *CO*. Between these regions of *CO* and hGATA1, twenty one percent of the amino acids are identical and 65% are similar or identical. Therefore although *CO* is not a member of the GATA1 family it shows similarity to them in the region of the zinc fingers and represents a new class of zinc-finger containing protein.

A further indication that these regions are important for *CO* activity is that the mutations in both the *co-1* and *co-2* alleles affect residues that are conserved between the proposed finger regions: *co-2* changes an arginine on the carboxy side of the N-terminal finger to a histidine, and the *co-1* deletion removes three amino acids from the carboxy side of the C-terminal finger.

Expression of CO mRNA in long and short day grown plants

No *CO* cDNA clones were found by screening several *Arabidopsis* cDNA libraries and the mRNA was not detected on Northern blots of polyA mRNA extracted from seedlings at the 3-4 leaf stage (data not shown). RT-PCR followed by Southern blotting and hybridisation to a *CO* specific probe was therefore used to detect the *CO* transcript. The RNA used in these experiments was isolated from seedlings at the 3-4 leaf stage, because this is just

before the floral bud is visible under long days and therefore seemed a likely time for the gene to be expressed.

Six independent RNA preparations made from plants growing under long days all produced a hybridising fragment of the size expected for the *CO* cDNA. No difference in abundance of the *CO* transcript was detected between wild-type or *co-1* mutant plants, suggesting that activity of the *CO* gene is not required to promote its own transcription.

Flowering time under long days is influenced by CO gene dosage.

Plants that are heterozygous for a wild-type allele and either *co-1* or *co-2* flower at a time intermediate between *co* homozygotes and Landsberg *erecta* under long days (Koorneef et al, 1991; F. Robson, unpublished). Sequencing of these mutant alleles demonstrated that they both contain in frame alterations to the amino acid sequence. This might suggest two models for the partial dominance of *co*. The mutant alleles might give rise to an altered product that interferes with floral induction, or the mutations might cause loss of function and the two-fold reduction in the level of the *CO* protein in a heterozygote lead to a delay in flowering time (haplo-insufficiency). The haplo-insufficiency explanation is favoured by the results included herein.

In the complementation experiments, transgenic

plants containing two copies of cosmids B or C and homozygous for the *co-2* allele often flowered at the same time as wild-type plants under long days. If the mutant allele encoded a product that interfered with the activity of the wild-type protein, then this would not be expected to occur. Moreover, the need to use RT-PCR to detect the *CO* transcript suggests that it is present at very low levels, which is consistent with the possibility that further reductions in transcript level causes late flowering.

Increases in the dosage of *CO* can lead to slightly earlier flowering under long days. This was concluded from the observation that some of the transgenic lines carrying extra copies of the *CO* gene flowered slightly earlier than wild type plants (Tables 1 and 2). This observation, together with the haplo-insufficiency phenotype discussed above, suggests that the level of expression of *CO* is a critical determinant of flowering time of *Arabidopsis* under long days.

METHODS

Growth conditions and measurement of flowering time

Flowering time was measured under defined conditions by growing plants in Sanyo Gallenkamp Controlled Environment rooms at 20°C. Short days comprised a photoperiod of 10 hours lit with 400 Watt metal halide power star lamps supplemented with 100 watt

tungsten halide lamps. This provided a level of photosynthetically active radiation (PAR) of 113.7 μmoles photons $m^{-2}s^{-1}$ and a red:far red light ration of 2.41. A similar cabinet and lamps were used for the long day. The photoperiod was for 10 hours under the same conditions used for short days and extended for a further 8 hours using only the tungsten halide lamps. In this cabinet the combination of lamps used for the 10 hour period provided a PAR of 92.9 μmoles photons m^{-2} s^{-1} and a red:far red ratio of 1.49. The 8 hour extension produced PAR of 14.27 μmoles m^{-2} s^{-1} and a red:far-red ratio of 0.66.

The flowering times of large populations of plants were measured in the greenhouse. In the summer the plants were simply grown in sunlight. In winter supplementary light was provided so that the minumum daylength was 16 hours.

To measure flowering time, seeds were placed at 4°C on wet filter paper for 4 days to break dormancy and were then sown on soil. Germinating seedlings were usually covered with cling film or propagator lids for the first 1-2 weeks to prevent dehydration. Flowering time was measured by counting the number of leaves, excluding the cotyledons, in the rosette at the time the flower bud was visible. Leaf numbers are shown with the standard error at 95% confidence limits. The number of days from sowing to the appearance of the flower bud was also recorded, but is not shown. The close correlation

between leaf number and flowering time was previously demonstrated for Landsberg *erecta* and *co* alleles (Koorneef et al, 1991).

Plant material

The standard wild-type genotype used was *Arabidopsis thaliana* Landsberg *erecta*. The *co-1* mutation was isolated by Redei (1962) and is in an *ERECTA* background, that in our experiments showed no detectable RFLPs or sequence variation from Landsberg *erecta*. The *co-2* allele was isolated in Landsberg *erecta* (Koornneef et al, 1991). The details of the lines used for the accurate RFLP mapping of *co* were described previously (Putterill et al, 1993).

In all cases described, lines carrying *co-2* also carried *tt4*, although in order not to over-complicate the genotype descriptions in the text this is not mentioned. The *tt4* mutation is within the chalcone synthase gene and prevents anthocyanin accumulation in the seed coat, but does not affect flowering time (Koornneef et al, 1983). The mutation is located on chromosome 5, approximately 3.3cM from *co* (Putterill et al, 1993). The use of a *co-2 tt4* line was useful in confirming that individual plants did carry the *co-2* mutation.

RNA extractions

RNA was extracted using a method which is a modified version of that described by Stiekma et al

(1988). Approximately 5 g of tissue frozen in liquid nitrogen was ground in a coffee grinder and extracted with a mixture of 15 ml of phenol and 15 ml of extraction buffer (50 mM Tris pH8, 1 mM EDTA, 1% SDS). The mixture was shaken, centrifuged and 25 ml of the aqueous layer recovered. This was then shaken vigorously with a mixture of 0.7 ml 4M sodium chloride, 10 ml phenol and 10 ml of chloroform. The aqueous layer was recovered after centrifugation and extracted with 25 ml of chloroform. The RNA was then precipitated from 25 ml of the aqueous layer by the addition of 2 ml of 10 M LiCL, and the precipitate recovered by centrifugation. The pellet was dissolved in 2 ml DEPC water and the RNA precipitated by the addition of 0.2 ml of 4M sodium chloride and 4 ml of ethanol. After centrifugation the pellet was dissolved in 0.5 ml of DEPC water and the RNA concentration determined.

DNA extractions

Arabidopsis DNA was performed by a CTAB extraction method described by Dean et al (1992).

Isolation of cDNA by RT-PCR

Total RNA was isolated from whole seedlings at the 2-3 leaf stage growing under long days in the greenhouse. For first strand cDNA synthesis, 10 μg of RNA in a volume of 10 μl was heated to 65^{0}C for 3 minutes, and then quickly cooled on ice. 10 μl of

reaction mix was made containing 1 μl of RNAsin, 1 μl of standard dT_{17}-adapter primer (1 μg/μl; Frohman et al, 1988), 4μl of 5x reverse transcriptase buffer (250mM TrisHCl pH8.3, 375mM KCl, 15mM $MgCl_2$), 2μl DTT (100mM), 1μl dNTP (20mM), 1μl reverse transcriptase (200 units, M-MLV Gibco). This reaction mix was then added to the RNA creating a final volume of 20μl. The mixture was incubated at 42°C for 2 hours and then diluted to 200 μl with water.

10μl of the diluted first strand synthesis reaction was added to 90μl of PCR mix containing 4μl 2.5mM dNTP, 10μl 10xPCR buffer (Boehringer plus Mg), 1μl of a 100ng/μl solution of each of the primers, 73.7μl of water and 0.3μl of 5 units/μl Taq polymerase (Boehringer or Cetus Amplitaq). The primers used were CO49 (5'GCTCCCACACCATCAAACTTACTAC 5' end located 38 bp upstream of translational start of *CO*) and CO50 (5'CTCCTCGGCTTCGATTTCTC 5' end located 57 bp upstream of translational termination codon of *CO*). The reaction was performed at 94°C for 1 minute, 34 cycles of 55°C for 1 minute, 72°C for 2 minutes and then finally at 72°C for 10 minutes.

20 μl of the reaction was separated through an agarose gel, and the presence of a fragment of the expected size was demonstrated after staining with ethidium bromide. The DNA was transferred to a filter, and the fragment of interest was shown to hybridise to a short DNA fragment derived from the *CO* gene. The

remainder of the PCR reaction was loaded onto another gel, the amplified fragment was extracted, treated with T4 DNA polymerase and ligated to Bluescript vector (Stratagene) cleaved with *EcoRV*. The PCR reaction was done in duplicate, and two independently amplified cDNAs were sequenced to ensure that any PCR induced errors were detected.

Isolation of cDNA fragments by 3' RACE

First strand cDNA synthesis was performed using the same conditions, RNA preparation and dT_{17}-adapter as described above for RT-PCR. The PCR was then performed using the standard adapter primer (5'gactcgagtcgacatcg; Frohman et al, 1988) and the CO49 primer described above. The PCR conditions were the same as described above, except that the amplification cycle was preceded by a 40 minute extension at 72°C. 20µl of the reaction was separated through an agarose gel, and a smear of fragments between 550 bp and 1.6 kb in length was detected. The remainder of the reaction was loaded on a similar gel, the region predicted to contain fragments of 1-2 kb was excised, the DNA extracted and subjected to a second round of PCR using the adapter primer and another *CO* specific primer (CO28, 5'tgcagattctgcctacttgtgc, 5' end located 94 bp downstream of translational start site of *CO*). When this PCR was monitored on an agarose gel a fragment around the expected size of 1.3 kb was detected. This

fragment was extracted from the gel, treated with T4 DNA polymerase and ligated to Bluescript DNA cleaved with *EcoRV*. Four amplified fragments recovered from two independent amplifications were sequenced entirely. All four were polyadenylated at slightly different positions, as described in the text.

Detection of CO transcript by RT-PCR

First strand synthesis was performed exactly as described above for the method used to isolate a cDNA clone, except that the RNA was isolated from plant grown in controlled environment cabinets at different stages. All samples were harvested and analysed in duplicate.

The primers used to amplify *CO* cDNA are described in the text and previously in Experimental Procedures. The primers used to amplify the cDNA of the gene used as a control were CO1 (5' TGATTCTGCCTACTTGTGCTC) and CO2 (5' GCTTGGTTTGCCTCTTCATC).

DNA sequencing

The Sanger method was used to sequence fragments of interest inserted in a Bluescript plasmid vector. Reactions were performed using a Sequenase kit (United States Biochemical Corporation).

Isolation of clones containing each of the seven co alleles

DNA was extracted from plants homozygous for each

of the alleles. Approximately 1ng of genomic DNA was diluted to 10μl with water and added to 90μl of reaction mix, as described above except that primers CO41 (5'ggtcccaacgaagaagtgc 5' end located 263 bp upstream of translational start codon of *CO*) and CO42 (5'cagggaggcgtgaaagtgt 5' end located 334 bp downstream of translational stop codon of *CO*) were used. The PCR conditions were: 94°C for 3 minutes, followed by 34 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes and then finally 72°C for 10 minutes. In each case this produced a major fragment of the expected size, 1.95 kb. The PCR was carried out in duplicate for each allele. In each case the reactions were extracted with phenol and chloroform, ethanol precipated and treated with T4 DNA polymerase. The reactions were then separated through an agarose gel, the fragment purified and ligated to SK+Bluescript cleaved with *EcoRV*. Ligations were introduced into *E.coli* DH5 alpha and the recombinant plasmids screened by colony PCR for those carrying an insertion of the expected size. The DNA sequences of two independently amplified fragments derived from each allele were determined.

Screening phage and cosmid libraries

A lysate of the cosmid library (Olszewski and Ausubel, 1988) was used to infect *E. coli* DH5 alpha, and twenty thousand colonies were screened with the probes described in the text. Three cDNA libraries were

screened to try to identify a *CO* cDNA. The number of plaques screened were $5x10^5$ from the "aerial parts" library (supplied by EC *Arabidopsis* Stock Center, MPI, Cologne), $3x10^5$ plaques of a library made from plants growing in sterile beakers (made by Dr A. Bachmair and supplied by the EC *Arabidopsis* Stock Center) and $1x10^6$ plaques of the CD4-71-PRL2 library (supplied by the *Arabidopsis* Biological Resource Center at Ohio State University).

Transformation of Arabidopsis

The cosmids containing DNA from the vicinity of *CO* were mobilised into *Agrobacterium tumefaciens* C58C1, and the T-DNA introduced into *Arabidopsis* plants as described by Valvekens et al, 1988. Roots of plants grown *in vitro* were isolated and grown on callus-inducing medium (Valvekens et al, 1988) for 2 days. The roots were then cut into short segments and co-cultivated with *Agrobacterium tumefaciens* carrying the plasmid of interest. The root explants were dried on blotting paper and placed onto callus-inducing medium for 2-3 days. The *Agrobacterium* were washed off, the roots dried and placed onto shoot inducing medium (Valvekens et al, 1988) containing vancomycin to kill the *Agrobacterium* and kanamycin to select for transformed plant cells. After approximately 6 weeks green calli on the roots start to produce shoots. These are removed and placed in petri dishes or magenta pots

containing germination medium (Valvekens et al, 1988). These plants produce seeds in the magenta pots. These are then sown on germination medium containing kanamycin to identify transformed seedlings containing the transgene (Valvekens et al, 1988).

EXAMPLE 2 - Construction of promoter fusions to the *CO* open reading frame:

A *PvuII-EcoRV* fragment containing the entire *CO* gene was inserted into the unique *EcoRV* site of the Bluescript™ plasmid. The *CO* gene fragment was inserted in the orientation such that the end defined by the *EcoRV* site was adjacent to the *HindIII* site within the Bluescript™ polylinker. This plasmid was called p*CO*1. The *PvuII-EcoRV* fragment inserted in p*CO*1 contains two *HindIII* sites both 5' of the point at which translation of the *CO* protein is initiated. Cleavage of p*CO*1 with *HindIII* produces a fragment that contains the entire *CO* open reading frame from 63bp upstream of the initiation of translation to the *PvuII* site which is downstream of the polyadenylation site, as well as all of the bluescript vector from the *PvuII/EcoRV* junction created by the ligation event to the *HindIII* site within the polylinker. Ligation of a promoter containing fragment in the appropriate orientation to this fragment creates a fusion of the promoter to the *CO* open reading frame. For instance, a variety of promoters may be inserted at

this position, as discussed below.

A GSTII promoter fusion to the CO open reading frame

The *GSTII* promoter-containing fragment was derived from plasmid pGIE7 (supplied by Zeneca) as a *HindIII-NdeI* fragment, whose sequence is shown in Figure 2. An oligonucleotide adapter (5' TACAAGCTTG) was inserted at the *NdeI* site to convert it into a *HindIII* site. The resulting plasmid was then cleaved with *HindIII*, and the promoter containing fragment ligated to the *HindIII* fragment containing the *CO* open reading frame. A recombinant plasmid that contained the *GSTII* promoter in the orientation such that transcription would occur towards the *CO* open reading frame was identified by *PstI* digestion. The GSTII-*CO* fusion was then moved into a binary vector described by Jones et al (1992) as a *ClaI-XbaI* fragment.

The binary vector may be introduced into an *Agrobacterium tumefaciens* strain and used to introduce the fusion into dicotyledonous species, or the fusion may be introduced into monocotyledonous species by a naked DNA transformation procedure. Protocols for transformation have been established for many species, as discussed earlier.

The GSTII promoter may be used to induce expression of the *CO* gene by application of an exogenous inducer such as the herbicide safeners dichloramid and flurazole, as described in WO93/01294 (Imperial Chemical

Industries Limited).

A heat shock promoter fusion to the CO open reading frame

An alternative inducible system makes use of the well characterised soybean heat shock promoter, Gmhsp17.3B, which is induced by expression in response to exposure to high temperatures in a variety of plant species (discussed by Balcells et al, 1994). The promoter is available as a 440 bp *XbaI-XhoI* fragment (Balcells et al, 1994) which after treatment with T4 DNA polymerase may be inserted into *pCO1* cleaved with *HindIII*, as described above for the GSTII fusion. The resulting fusion may then be introduced into the binary vector, *Agrobacterium tumefaciens* and transgenic plants, as described earlier. *CO* expression may be induced by exposing plants to temperatures of approximately 40°C.

Fusion to the CO gene of a modified CaMV 35S promoter containing tetracycline resistance gene operators

A modified CaMV 35S promoter which contains three operators from the bacterial tetracycline resistance gene has been developed as a chemically inducible system. In the presence of the tetracycline gene reporessor protein this promoter is inactive, but this repression is overcome by supplying plants with tetracycline (Gatz et al, 1992). This is an alternative chemically inducible promoter which may be

fused to the *CO* open reading frame. The promoter is available as a *SmaI-XbaI* fragment (Gatz et al, 1992) which after treatment with T4 DNA polymerase may be inserted into *pCO1* cleaved with *HindIII* as described earlier. After introduction of this fusion into plants also containing the repressor gene, *CO* expression may be induced by supplying the plants with tetracycline.

A CaMV 35S promoter fusion to the CO open reading frame

The CaMV 35S promoter was isolated from plasmid pJIT62 (physical map of which is shown in Figure 4). The *KpnI-HindIII* fragment containing the CaMV 35S promoter was fused to the *CO* open reading frame by ligation to plasmid *pCO1* cleaved with *HindIII* and *KpnI*. The single *KpnI* site was then converted to a *ClaI* site by insertion of an adapter oligonucleotide (5'TATCGATAGTAC), and then a *ClaI-BamHI* fragment containing the promoter fused to the *CO* ORF was inserted into a binary vector. The fusion may be introduced into transgenic plants either by the use of *Agrobacterium tumefaciens* or as naked DNA, as described earlier.

Fusion of the meri 5 promoter to the CO open reading frame

The meri 5 promoter is available as a 2.4 kb *BglII-StuI* fragment (Medford et al, 1991). This may be treated with T4 DNA polymerase and inserted into the *HindIII* site of *pCO1* as described above. The fusion may

then be introduced into transgenic plants, as described above.

EXAMPLE 3 - Flowering time under short days of plants carrying extra copies of *CO*

Under short day conditions wild type plants and *co-2* homozygotes both flower at approximately the same time (Table 1), suggesting that the *CO* product is not required for flowering under these conditions. However, under short days, several of the *co-2 tt4* families carrying the T-DNAs derived from cosmids B and C flowered earlier than both the parental *co-2* line and wild type (Table 1). In particular, 2 lines (4 and 6) carrying cosmid C flowered much earlier than wild type. This suggested that in some families a transgenic copy of *CO* was expressed at a higher level than the original copy, or expressed ectopically, and that this led to earlier flowering under short days than that of wild type plants.

Cosmid B was also introduced into wild-type Landsberg *erecta* plants and T2 plants homozygous for the transgene at a single locus were identified in the same way as described above (Table 1). Of the 3 independent transformants analysed in the T3 generation, one flowered slightly earlier than wild-type plants under long days, and significantly earlier under short days (Table 1). This again suggested that at least at some chromosomal locations, extra copies of the *CO* gene can

cause early flowering.

EXAMPLE 4 - Influencing flowering characteristics using a CaMV 35S promoter/CO gene fusion

A fusion of a CaMV 35S promoter to the *CO* open reading frame was introduced into *co* mutant *Arabidopsis* plants. First the *ClaI-BamH1* fragment described in Example 2 was inserted into the *ClaI-BamH1* sites of binary vector SLJ1711 (Jones et al., 1992). An *Agrobacterium tumefaciens* strain carrying this vector was then used for transformation of *Arabidopsis* root explants, followed by regeneration of transformed plants as described by Valvekens et al. (1988).

The resulting transgenic plants flowered significantly earlier than wild-type under both inductive and non-inductive conditions. For example, under inductive long-day conditions, wild-type plants flowered after forming approximately 5 leaves, while the transgenic plants flowered with 3-4 leaves. Under non-inductive short days, wild-type plants flowered with approximately 20 leaves, while the transgenic plants formed 3-4 leaves. The use of promoter fusions to increase the abundance of the *CO* mRNA, or to alter the specificity of *CO* transcription, can therefore be used to lead to dramatically earlier flowering than that of wild-type plants.

In addition, some of the transgenic plants carrying the fusion of the CaMV 35S promoter to the *CO* gene

formed a terminal flower at the end of the shoot. The shoot of wild-type plants shows indeterminate growth, growing and forming flowers on the sides of the shoot indefinately. However, *terminal flower (tfl)* mutants show determinate growth, terminating shoot development prematurely by forming a flower at the apex of the shoot. In wild-type plants, the *TFL* gene is thought to prevent the formation of flowers at the apex of the shoot, by preventing the expression of genes that promote flower development, such as *LEAFY (LFY)*, in the apical cells. This is supported by the observations that *LFY* is expressed in the shoot apex of *tfl* mutants but not wild type plants, and that fusions of the CaMV 35S promoter to *LFY* cause transgenic plants to form a terminal flower (Weigel and Nilsen, 1995). While not intending to be bound by any particular theory, the fusion of *CO* to the CaMV 35S promoter might therefore cause a terminal flower by activating genes such as *LFY* at the apex of the shoot.

The two phenotypes caused by the *CO* fusion to the CaMV 35S promoter, early flowering and the formation of a terminal flower, may be separated by the use of other promoters. For example, terminal flower formation might be optimised by using a promoter, such as that of the meri 5 gene mentioned above, that is expressed mainly in the apical meristem, while early flowering without a terminal flower might result from expressing the gene from the promoters that are not well expressed in the

apical meristem, such as a heat-shock promoter.

Example 5 - Cloning of a *CO* homologue from *Brassica napus*

Low stringency hybridizations (Sambrook et al., 1989) were used to screen a lambda genomic DNA library made from *Brassica napus* DNA. Positively hybridizing clones were analysed and classified by constructing maps of their restriction enzyme cleavage sites (using *HindIII*, *XhoI*, *EcoRV*, *XbaI*, *EcoRI* and *NdeI*) *CO* homologues were distinguished from other members of the *CO* gene family because of the similarity of their restriction enzyme map with that of the *Arabidopsis CO* gene, and because a second gene that is located close to *CO* in the *Arabidopsis* genome was shown to be present at a similar position in the *Brassica* clones. Two *CO* homologues, corresponding to the genes present on *Brassica napus* linkage groups N10 and N19 (Sharpe et al., 1995), were then sub-cloned into plasmids and sequenced. The sequence of the gene from the N10 linkage group is shown in Figure 5 and that from the N19 linkage group is shown in Figure 6. The amino acid sequences of the proteins encoded by these genes are very similar to that of the *Arabidopsis CO* gene, particularly in the regions demonstrated by mutagenesis to be important for the functioning of the protein; 86 amino acids across the zinc-finger region are 84% identical, and a 50 amino acid region at the carboxy

terminus of the protein, that is affected in two of the *Arabidopsis* mutants, is 88% identical. These two regions are the most conserved, with the intervening 187 amino acids from the middle of the protein being 64% identical.

This sequence analysis indicates that *CO* homologues can be isolated from plant species other than *Arabidopsis*. In addition, restriction fragment length polymorphism mapping strongly suggests that *CO* homologues are important in regulating flowering time of other species. For example, in *Brassica nigra* a *CO* homologue closely co-segregates with a major quantitative trait locus for flowering time (U. Lagercrantz et al, in press), and in *Brassica napus CO* homologues mapping to linkage groups N2 and N12 co-segregate with allelic variation for flowering time.

TABLE 1 - Flowering time and segregation of kanamycin resistance in T2 and T3 generations of *co-2* carrying the T-DNA of cosmid B or C plants

Trans-genic *co tt4* line scored	Ratio of Km resistant seedlings in T2[1]	Average LN at flowering of T3 individual under LDs[2]	Average LN at flowering of T3 individual under SDs[2]	Ratio of Km resistant seedlings in T3
cosmid B line 1	3:1	4.6+/-0.4	14.0+/-2.5	1:0
cosmid B line 2	3.7:1	4.2+/-0.3	18.5+/-1.1	1:0
cosmid B line 3	2.9:1	4.6+/-0.8	13.5+/-4.1	1:0
cosmid B line 4	2.4:1	4.6+/-0.8	16.4+/-2.2	1:0
cosmid B line 5	3.0:1	5.1+/-0.5	18.5+/-1.1	1:0
cosmid C line 1	2.9:1	4.6+/-0.6	20.6+/-3.8	1:0
cosmid C line 2	3.4:1	3.9+/-0.4	11.7+/-3.2	1:0
cosmid C line 3	3.3:1	4.0+/-0.4	20.4+/-1.2	1:0
cosmid C line 4	4.9:1	3.7+/-0.3	[3]7.6+/-5.3	1:0
cosmid C line 5	3:1	4.9+/-0.6	17.7+/-2.1	1:0
cosmid C line 6	3.8:1	3.5+/-0.5	6.6+/-1.4	1:0
Landsberg *erecta*	-	5.1+/-0.8	18.9+/-2.4	-
co-2	-	12.4+/-1.0	18.1+/-3.4	-

Flowering time was measured by counting the number of leaves present at the time that the flower bud appeared

in the centre of the rosette (Koornneef et al, 1991; Experimental Procedures).

[1] Over 80 plants were tested in each family, except for cosmid B line 3 in which 35 plants were used.

[2] 10 plants from each family were tested

[3] The large standard error in this population was due to 2 plants that flowered with 18 leaves, while the other 8 has a leaf number of 5.1+/-1 at flowering. Southern analysis of this line using a T-DNA fragment as probe identified 6 hybridising fragments. The variation in flowering time could therefore be due to the segregation of one T-DNA copy that is required for early flowering, or to the occurrence of co-suppression repressing activity of the transgenes in some individuals.

TABLE 2 - Flowering time of transgenic wild-type plants carrying extra copies of the *CO* gene

Landsberg *erecta* transgenic line	Km in T2[1]	Average LN at flowering of T3 individuals under LDs[2]	Average LN at flowering of T3 individuals under SDs[2]	Ratio of kanamycin resistance in T3
cosmid B line 1	3.4:1	4.4+/-1.0	18.1+/-2.1	1:0
cosmid B line 2	5.9:1	3.2+/-0.6	10.1+/-2.2	1:0
cosmid B line 3	2.8:1	4.0+/-0.5	19.6+/-2.2	1:0
Landsberg *erecta*		5.1+/-0.8	18.9+/-2.4	-
co-2		12.4+/-1.0	18.1+/-3.7	-

[1] Over 80 plants were tested in each family.

[2] 10 plants from each family were tested.

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CLAIMS

1. A nucleic acid isolate comprising a nucleotide sequence encoding a polypeptide with CO function.

2. Nucleic acid according to claim 1 wherein said nucleotide sequence is that of the CO gene of *Arabidopsis thaliana* or a CO homologue from another plant species, or a mutant, derivative or allele of the gene or homologue.

3. Nucleic acid according to claim 2 wherein said CO nucleotide sequence is shown in Figure 1.

4. Nucleic acid according to claim 2 wherein said CO homologue is from *Brassica*.

5. Nucleic acid according to claim 4 wherein said CO homologue nucleotide sequence is shown in Figure 5 or Figure 6.

6. Nucleic acid according to claim 1 or claim 2 wherein expression of said nucleotide sequence delays flowering in a transgenic plant.

7. Nucleic acid according to claim 2 wherein the polypeptide encoded by said nucleotide sequence is a mutant or derivative of wild-type CO or a wild-type CO homologue and expression of said nucleotide sequence

delays flowering in a plant.

8. Nucleic acid according to claim 2 wherein the polypeptide encoded by said nucleotide sequence is a mutant or derivative of wild-type CO or a wild-type CO homologue and expression of said nucleotide sequence promotes flowering in a plant.

9. Nucleic acid according to claim 1 or claim 2 wherein expression of said nucleotide sequence promotes flowering in a transgenic plant.

10. A nucleic acid isolate comprising a nucleotide sequence encoding a polypeptide able to complement a mutant phenotype in a plant, which phenotype is delayed flowering, the timing of flowering being substantially unaffected by vernalisation.

11. A nucleic acid isolate comprising a nucleotide sequence which is a mutant or derivative of a wild-type gene encoding a polypeptide with ability to influence the timing of flowering, the mutant or derivative phenotype being delayed or early flowering with the timing of flowering being substantially unaffected by vernalisation.

12. Nucleic acid according to any of claims 1 to 11 further comprising a regulatory sequence for expression of said polypeptide.

13. Nucleic acid according to claim 12 comprising an inducible promoter.

14. Nucleic acid according to claim 13 wherein the promoter is derived from a maize gene for a 27 kD sub-unit of glutathione-S-transferase, isoform II.

15. A nucleic acid isolate comprising a nucleotide sequence complementary to a coding sequence of any of claims 1 to 11 or a fragment of a said coding sequence.

16. Nucleic acid which is DNA according to any one of claims 1 to 12 or claim 15 wherein said nucleotide sequence or a fragment thereof is under control of a regulatory sequence for anti-sense transcription of said nucleotide sequence or a fragment thereof.

17. Nucleic acid according to claim 16 comprising an inducible promoter.

18. Nucleic acid according to claim 17 wherein the promoter is derived from a maize gene for a 27 kD sub-unit of glutathione-S-transferase, isoform II.

19. A nucleic acid vector suitable for transformation of a plant cell and comprising nucleic acid according to any one of the preceding claims.

20. A plant cell comprising nucleic acid according to

any preceding claim.

21. A plant cell according to claim 20 having heterologous said nucleic acid within its genome.

22. A plant cell according to claim 21 having more than one said nucleotide sequence per haploid genome.

23. A plant comprising plant cell according to any one of claims 20 to 22.

24. Selfed or hybrid progeny or a descendant of a plant according to claim 23, or any part or propagule of such a plant, progeny or descendant, such as seed.

25. A method of influencing a flowering characteristic of a plant, the method comprising causing or allowing expression of the polypeptide encoded by nucleic acid according to any one of claims 1 to 14 from that nucleic acid within cells of the plant.

26. A method of influencing a flowering characteristic of a plant, the method comprising causing or allowing transcription from nucleic acid according to any one of claims 1 to 14 within cells of the plant.

27. A method of influencing a flowering

characteristic of a plant, the method comprising causing or allowing anti-sense transcription from nucleic acid according to any one of claims 15 to 18 within cells of the plant.

28. A method of identifying and cloning CO homologues from plant species other than *Arabidopsis thaliana* which method employs a nucleotide sequence derived from that shown in Figure 1.

29. Nucleic acid encoding a CO homologue obtained by the method of claim 28.

30. Nucleic acid according to claim 29 which comprises a nucleotide sequence shown in Figure 5 or Figure 6.

31. A method of identifying and cloning CO homologues from plant species other than *Arabidopsis thaliana* which method employs a nucleotide sequence derived from a sequence shown in Figure 5 or Figure 6.

32. Nucleic acid encoding a CO homologue obtained by the method of claim 31.

Figure 1.

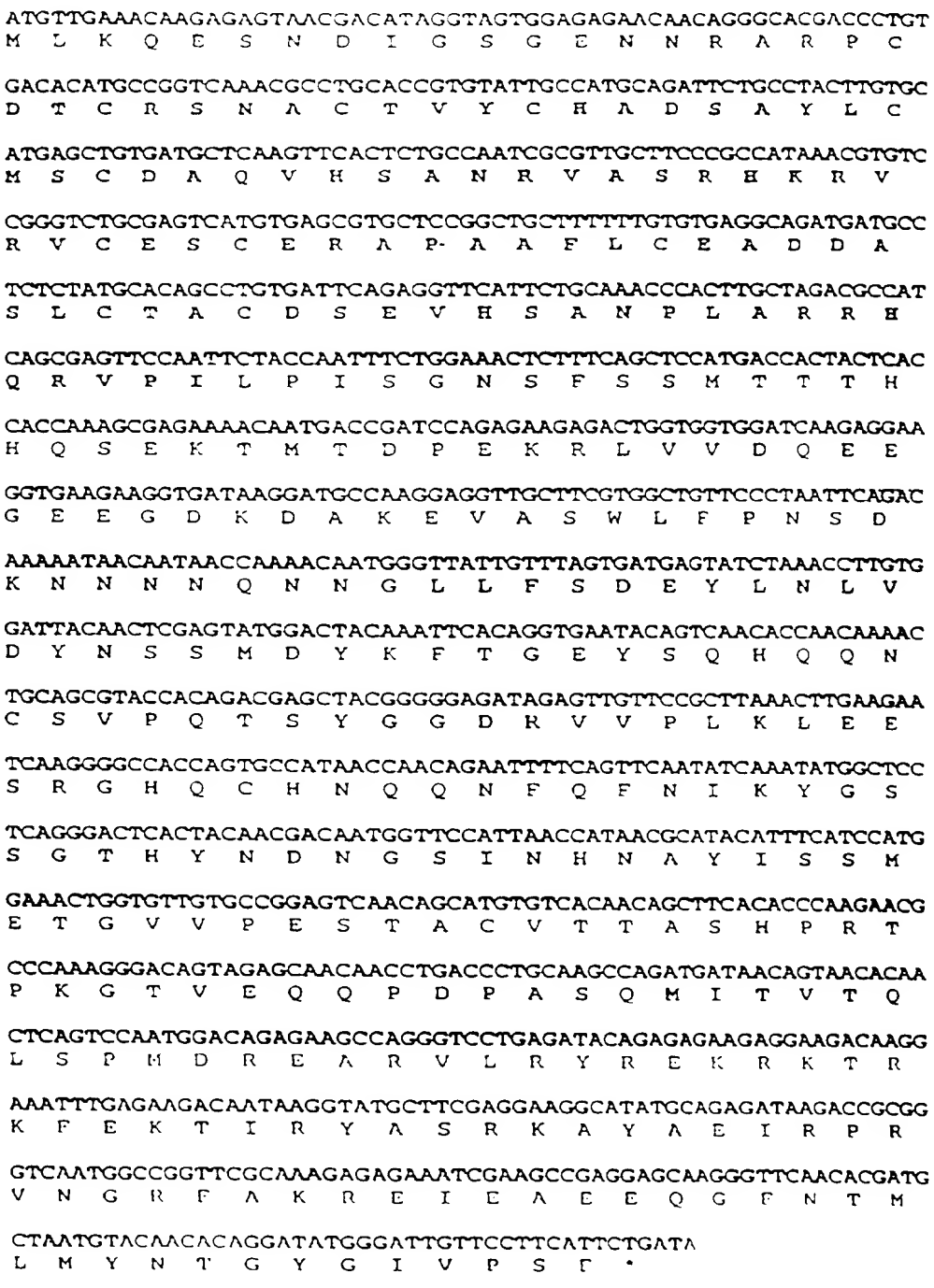
1 ATGTTGAAACAAGAGAGTAACGACATAGGTAGTGGAGAGAACAACAGGGCACGACCCTGT
M L K Q E S N D I G S G E N N R A R P C
61 GACACATGCCGGTCAAACGCCTGCACCGTGTATTGCCATGCAGATTCTGCCTACTTGTGC
D T C R S N A C T V Y C H A D S A Y L C
121 ATGAGCTGTGATGCTCAAGTTCACTCTGCCAATCGCGTTGCTTCCCGCCATAAACGTGTC
M S C D A Q V H S A N R V A S R H K R V
181 CGGGTCTGCGAGTCATGTGAGCGTGCTCCGGCTGCTTTTTTGTGTGAGGCAGATGATGCC
R V C E S C E R A P- A A F L C E A D D A
241 TCTCTATGCACAGCCTGTGATTCAGAGGTTCATTCTGCAAACCCACTTGCTAGACGCCAT
S L C T A C D S E V H S A N P L A R R H
301 CAGCGAGTTCCAATTCTACCAATTTCTGGAAACTCTTTCAGCTCCATGACCACTACTCAC
Q R V P I L P I S G N S F S S M T T T H
361 CACCAAAGCGAGAAAACAATGACCGATCCAGAGAAGAGACTGGTGGTGGATCAAGAGGAA
H Q S E K T M T D P E K R L V V D Q E E
421 GGTGAAGAAGGTGATAAGGATGCCAAGGAGGTTGCTTCGTGGCTGTTCCCTAATTCAGAC
G E E G D K D A K E V A S W L F P N S D
481 AAAAATAACAATAACCAAAACAATGGGTTATTGTTTAGTGATGAGTATCTAAACCTTGTG
K N N N N Q N N G L L F S D E Y L N L V
541 GATTACAACTCGAGTATGGACTACAAATTCACAGGTGAATACAGTCAACACCAACAAAAC
D Y N S S M D Y K F T G E Y S Q H Q Q N
601 TGCAGCGTACCACAGACGAGCTACGGGGGAGATAGAGTTGTTCCGCTTAAACTTGAAGAA
C S V P Q T S Y G G D R V V P L K L E E
661 TCAAGGGGCCACCAGTGCCATAACCAACAGAATTTTCAGTTCAATATCAAATATGGCTCC
S R G H Q C H N Q Q N F Q F N I K Y G S
721 TCAGGGACTCACTACAACGACAATGGTTCCATTAACCATAACGCATACATTTCATCCATG
S G T H Y N D N G S I N H N A Y I S S M
781 GAAACTGGTGTTGTGCCGGAGTCAACAGCATGTGTCACAACAGCTTCACACCCAAGAACG
E T G V V P E S T A C V T T A S H P R T
841 CCCAAAGGGACAGTAGAGCAACAACCTGACCCTGCAAGCCAGATGATAACAGTAACACAA
P K G T V E Q Q P D P A S Q M I T V T Q
901 CTCAGTCCAATGGACAGAGAAGCCAGGGTCCTGAGATACAGAGAGAAGAGGAAGACAAGG
L S P M D R E A R V L R Y R E K R K T R
961 AAATTTGAGAAGACAATAAGGTATGCTTCGAGGAAGGCATATGCAGAGATAAGACCGCGG
K F E K T I R Y A S R K A Y A E I R P R
1021 GTCAATGGCCGGTTCGCAAAGAGAGAAATCGAAGCCGAGGAGCAAGGGTTCAACACGATG
V N G R F A K R E I E A E E Q G F N T M
1081 CTAATGTACAACACAGGATATGGGATTGTTCCTTCATTCTGATA
L M Y N T G Y G I V P S F *

Figure 2.

```
   1  AAGCTTGGGC GTAGGTGTTG TCTATCGGCG AAAACACGCG CGGTACGCCA
  51  AGAACAGCGC GGCCATCTCC ATCCCAGGCA CGGTGCGCCC GCTTTTTCGC
 101  CGTCTCGCTG AGTCACGGCG GGCGTCCAGC AGGTAGTTGA GCGCCTTCCG
 151  CGGCACGAAT CGCTGCGTGC GGCCCGGATC TGGTCGAGTT GGTAGTCAGC
 201  GTCGGTGTCG AATGCCGGGA CGTCGACCAG GAAGAAGTTG CCGTCGCTGG
 251  GGTGGGGACG GAAGGCGTCA GGATTGTCGC AAGGGCAGAG CCCAGCCTGC
 301  GGGCGGGGCT ACCTCGTCGA CGCCTCGGCA CGGCGGCGGC AAAGCTGCTG
 351  CGGGACGTGC CCGCCTGGGC CGCCTTCTCG GTGAAGTGGT CCTCGAAGGG
 401  GACGAGCTCG CTGGGGTCAA ACCACCCCAT AGCTCGAGTC ACCGAAGAAG
 451  GCGACGAGGA CGAGCCCGTC GCGGTGGCCG CGGTGTACCT CCTCGTCGTC
 501  GGTGAGGCTG ACGCTGTAGA TATGGCCAGG CCACCACGGA TGGGACTTCA
 551  CCTTGGCCCA GACCATGTCG CCGAACCGGG GGCCGCCGTT CGCCCATGCG
 601  ATGCCGCGTC CGGCAGCAGG AACCATGGCG CCTCCAGCGG CGGGGTCGGA
 651  CATCCTGTGG AGGGGAACCG AAAACCTAGA TTTGGATGCA GGTTCGATTG
 701  GTCTGGGCTT GGGTTTGGGT TCCGGAGGAG GGTGGCCTGG GATCGGTGGA
 751  AGGAGGGACA TTGTTGGTAA TTTTTATTAT TTTATAATAT GGAGAAATTC
 801  GAGAGACTGA ACGATGGTGA TGTTTATTTG AGGACTATGT AGTATAAAGT
 851  GTAAAATAGT ATTTTATCAA GTTTATATTC ACGTTTTTGC TGAAGATAGT
 901  ATAATAGTGG AGTTGTTTTT GGCGGCTACA TAATCTTAGG CTATCTTCTC
 951  GGTCGCTCTC ATATCATATC TACTATCACA TTCTCTATTT TAAATTTCAC
1001  TTTGTGTAAT CTACACTATA AAATAGTGTT TTACACGGTA TGTTGTACAC
1051  AGCCTTATCG TGGCGCGACG GAGTTGGATA GAGATGGTGA ACAGCTGGAT
1101  AGATATGATT TATAGGCGAT TGGGTAGATG TGATTTGATA GGTGGTTATG
1151  TAGGAGCGAT TTAGTGAGAC ATTGTAAATA ATTAGGTTGA TGTGATCCGA
1201  GGATGGCTAG GTAGATATGA TTTTAATGGA TGGTTTGGTG GACTAAGTTA
1251  TGTGGACATT ATAATATGTT TTAAATTTCT AAGAAATTGT TTGTGTTAAA
1301  TTGTATCCCA CATAGATTAT TTAGCCATCT CAAAGAGAGG TTTGGGTTGT
1351  TTACACAAAT AAAATATTCG TTTGCTTCTA CAATTTATAT GTTTTTTATT
1401  TACATGAAAA CTATATTTTT TATTCATCTA CTCACCCAGC ACAGAAATTC
1451  TGGTTGAGTA GATGAAAAAA AACTACAACA AACTCTTCCT GAAAGTGTCG
1501  GTGTGAAGCC GAGAAATCCT TTTCATTTCG GTGACGGAGC CCCTTGCTGG
1551  CTGCTGCTCA GTGCACTCCG TTCGCCTGCC TGCCACTACA AGCGACGGCC
1601  GACGACTCGC AAGTATCGGT AGGCATTTTA AAACTGAAAA CCAAATCTAA
1651  ACCCGAATAG ACCAAAATTGT TGGTTTATTC GGGTTTTTGG GTTCGGATTC
```

```
1701  GGTTTCTAAA TATGCTATAT TTTAGGGTAT AGGTTCGGGT TCAGTTTCTA
1751  ACCTTTAAAA CCTGAATAGA CGAATAACCC GAAATATAAA AAATCTCTTA
1801  ATATGTGATG ATATTATTAT ATGATTTATG AACTTATTAA CCGAAAATAA
1851  TGATACCATC CTAACGATAG TATATATATC TATGTATGCT ATTTTTATAG
1901  TCACTTGTTG TAATAATAGT ACTTCCAATT AATTAATCAG TGTATATATT
1951  TTAACAAAAG ATACTAGCCT CTCTACTATT TGAGTATATT CGGTGCACCG
2001  AATAGACCGA ACCGAAATTG TAAGTCTATT CAGGTTCGGT TCCTAAAATT
2051  ATTTTAAAAA TTTTGGTTCT CATATTTCAG AATCCGAAAT TTCATAAATC
2101  CAAATAGACC GAACCAAATT ACGCTAATAG ACCGAATAAC TAGCGTACTC
2151  GCAAGTCGCA CCCCACTAGC CTGCTGCGTG CGTAAGCGAG GACGTCACGC
2201  GTTCTCCCTC CCGTCGACCA AATACACTTG GTCTTCTAGC ACCTTCTTCC
2251  TCTCCAAGAC TCCAATCCCC CAACCACCAG AACCAGCGCC AGCTCTAACG
2301  TCACCTCTGA TTTCTCTCTC CTCTCTATTG CTAGCTGCTT TATTATAAGT
2351  AGCAGCTGCA GCAGGCAGGA GCTGCACACA CCCATCCAAT TCCAGCTGCT
2401  GATCTTGATC CTGCACCCCG AGCCGTACAC AAGAGCTAGT CGGTAGAACT
2451  TGCAGGAGCG GAGCAGAACT AAGTGCAGAG AACAGGACAT ATG
```

Figure 3.

```
   1  GAATTCATGT ACCAAATCAA TACTTTTTAG CCATAAATGA GTCAGTTTTA
  51  GTATCCACAT GAATTTACCT ACCAGAGTGT TGTAAATTAT GTTCTTTTGG
 101  GGCCACTTAC ATGGATCTCA TTCATTCACT GCAGCGAGTT CTCAGACCAC
 151  CAGAAAATTT ATTCAGTGAT CTGTTTTGAT CATGCAACAT AAACTTATAA
 201  GCCACACAAG CAAAACAAAG ATATCCCATG TTGCATATAA TACGAGCTAG
 251  CATATCATAA AGAAGGAAAC TTGAAGTAGC AAAGTTTCTA CTAAATTTCT
 301  TGTCAGGAAT TTTTAAAATG CAATGACAAC CACTTGGAGC ACTATGAGTT
 351  TCAGAGCCAA TAGAATGTTA CTATTTGGTG TGGATTCGAG CTAGCACGTG
 401  AAAGTGCATA AAAGTGATTA CCTTTTGCCA AAGGTCACTG CACTTTTCCT
 451  CAGATAGTTT CTCACAGCCA TGGAAAGTGG AGAATCCGCA TAAACGTACA
 501  ATTACAAGCT TTATATGGTC CCTCGACTCT TATTCTCTTC TCAGTCTTTG
 551  CAACTAAATA GGGTTTTCGT TAATCTGAAA GAAGCAAAGT ATTCGAAACC
 601  ACGGAAACCT GATAAAGAAT GAAAACAAAT AAGCAATAGT GTTTTCTTGA
 651  AAATCTCGAT GCAACTTTGA GGATATTGTT ACATATGATC TATTACTCGT
 701  AACAGTTATC CGAAGGCCTA CACATGTGAG AGAAGTTCCA AACCGCTACA
 751  ACAATAAACT TAATTAGAGA CTGTCAACGA GCAATAATAA GCAAAACTAC
 801  TTTTTTCTTG AGCTACAAGT GAAAAGGCCA ATACACAATT TACTCTTCAT
 851  GAACTCGAAC CACGTTACAA TCTCCAAAAA ATTTCATCAC CAAAGCACTA
 901  AAAGCCAAAG ATGCCTCAAC TTATCCAACT TGGCAGGATA AAGATCTCCA
 951  AAAATGCTTA CTAAAGAACC TAGAATCTTT TCTTTAGAAT TCAATGATCA
1001  TATAACCATT TCATAACAAT TCTAAATGCC ATTACATTCA TCGTAAAACC
1051  AGTAAATAAC AAGAACTTGT ATGTTAAGTT CCAATTACCA AGCAAAAAAA
1101  AACTTTTCAA AGTTTAAAGT TCAAAATGGG AAAGAGAAGT GCGGTGTAAG
1151  CAAATATGAA AGAGGAAGAG ATGCGAAAAG TGTATCCTAG GACCAGCATT
1201  TTATACAAAA AAAAAACACT CACTTTTCAG CTCTTAAGGC ATAGAGTGAA
1251  GGTAGCCATA TGAATTTGGC CACTAGAGCG TCCGTCAAAT CTCATTCTTT
1301  TTGGACCACA TAATGGGTAT CATACATTCA CTGGACCCAA AAGCGTAACT
1351  GGAGCTAGTC CTCAAACCTA GAGAGTATCG TATCCTGTAG CTTCCACATA
1401  GTAAACATTA TGAGCATAAC ACCAACAAGG CAACTCCAAG TACTAATGGT
1451  TATTAGTACA GGAAAACCCA CGATGCTAAA CACATGAATG GGTCACCAAA
1501  TAGAGTGAAG ATGGTTAAAT TGCATCTATG GATCATGTGG ACTAGTAAAT
1551  GAGTGTAGCA GAAAACTTCA CAATTACCTC TGTGATCTTA GAAACATGTC
1601  CTGAAAATTC CATACAAGTG TCGTTTGTAT TAGATTACTT CCACAGGTTG
```

1651 AGATCTAATA AAGCTACAAT AAATAGTATA GAGTATCATC ATAAACCCAA
1701 ATTACAGAGA TGTGACAACA CTCATGAGTC ATGTTTTGTA ACTACTTACT
1751 ATAATGGTTA CCAAGTGCAA ATTTCTACAT ACTATATATG ATAAATCTAA
1801 TTATTGCTCA TGTGGACTCC AAAATGCCTT TTAAGTTTTA ACTTGTGCGT
1851 CAGGTAAATT CTAATTTGTA GTCTCAAGAC TACTTGGCGG ATTCGAGTTT
1901 GATCCTAGAA AATCCACCGT CTCTATGTTT TTCATGTCAC TTTTCCGATA
1951 TGATTCTCAT TACCATGACT TTATGAACCA GATTAAACAT TATAACACTT
2001 TTCATCAGAA AATCCTTCGA AAGTTTCAAT TGCAAATCTT TCTAAATGAT
2051 GCAGATGCAT TCACAAATAA TGGAACAACA ACTATACCAT ATTCACGAGT
2101 TTGTCTAACC TTTGTATAGG TAGTCAACCC ATAACAGTTG GTGATGGCTC
2151 TGACACTCGA AGCCTTACTC GGAGAGATAC CTGAACAGTA ATCACAAGGT
2201 TCAGGATGAA TATTCAACCA CTTAAACTTT GTATAAAGCC AAAGAGATAA
2251 AACGAATCTA GCTTTACTTT AAATAAAATG CATATGAAAA TAGTAAAAGG
2301 TGATACGAAA AAATAGTAAC AATTTGCCTG CAACACCATG GCATTATCCG
2351 GACCACTTCC TCTTGAGAAT CTCAGTATGG CAAGTGGCAA AACCTAAGCA
2401 ACTTGTGAAC GGGTCCCAAC GAAGAAGTGC ATAGGAGGAG ATGTTTACAC
2451 TTTACACTTT ACACTTTACA CTTTACACAT AGGCCTTCCC AAAAGCTCAA
2501 CTAGCTGCAA GAGGATCCAA TAACATGTAA GAGCCACTAA CGCTGTGCCA
2551 CGTGTAGGCA CTCAGGATTC GATCTTCCCC TCTACTTATT CTCTCACACC
2601 AGATATAAGC TTTATTAGCC CCTTCTTTCA GATACCAGCT CCCACACCAT
2651 CAAACTTACT ACATCTGAGT TATTATGTTG AAACAAGAGA GTAACGACAT
2701 AGGTAGTGGA GAGAACAACA GGGCACGACC CTGTGACACA TGCCGGTCAA
2751 ACGCCTGCAC CGTGTATTGC CATGCAGATT CTGCCTACTT GTGCATGAGC
2801 TGTGATGCTC AAGTTCACTC TGCCAATCGC GTTGCTTCCC GCCATAAACG
2851 TGTCCGGGTC TGCGAGTCAT GTGAGCGTGC TCCGGCTGCT TTTTTGTGTG
2901 AGGCAGATGA TGCCTCTCTA TGCACAGCCT GTGATTCAGA GGTTCATTCT
2951 GCAAACCCAC TTGCTAGACG CCATCAGCGA GTTCCAATTC TACCAATTTC
3001 TGGAAACTCT TTCAGCTCCA TGACCACTAC TCACCACCAA AGCGAGAAAA
3051 CAATGACCGA TCCAGAGAAG AGACTGGTGG TGGATCAAGA GGAAGGTGAA
3101 GAAGGTGATA AGGATGCCAA GGAGGTTGCT TCGTGGCTGT TCCCTAATTC
3151 AGACAAAAAT AACAATAACC AAAACAATGG GTTATTGTTT AGTGATGAGT
3201 ATCTAAACCT TGTGGATTAC AACTCGAGTA TGGACTACAA ATTCACAGGT
3251 GAATACAGTC AACACCAACA AAACTGCAGC GTACCACAGA CGAGCTACGG
3301 GGGAGATAGA GTTGTTCCGC TTAAACTTGA AGAATCAAGG GGCCACCAGT
3351 GCCATAACCA ACAGAATTTT CAGTTCAATA TCAAATATGG CTCCTCACGG

3401 ACTCACTACA ACGACAATGG TTCCATTAAC CATAACGTAA GGCTTTTGTA
3451 TATTTGTTAC CCCTTCAATT TAGCATCTTC CCATAACGCA GCAGGGTGAA
3501 TTCTTTCATC ATACACACAA ATCCACTGAT CCACTGCCAA CAGTTGATCT
3551 ATAGCACATA GAAATTTCAC CAGAAGTCTA TAATAAAAAC AATATATGCT
3601 TCCTTTTGCA TCGACTCTCT TTAGTCCTCT TACCAGGGGG ATTGAGAATG
3651 TCTTTGTTTC TGTCATTAGG CATACATTTC ATCCATGGAA ACTGGTGTTG
3701 TGCCGGAGTC AACAGCATGT GTCACAACAG CTTCACACCC AAGAACGCCC
3751 AAAGGGACAG TAGAGCAACA ACCTGACCCT GCAAGCCAGA TGATAACAGT
3801 AACACAACTC AGTCCAATGG ACAGAGAAGC CAGGGTCCTG AGATACAGAG
3851 AGAAGAGGAA GACAAGGAAA TTTGAGAAGA CAATAAGGTA TGCTTCGAGG
3901 AAGGCATATG CAGAGATAAG ACCGCGGGTC AATGGCCGGT TCGCAAAGAG
3951 AGAAATCGAA GCCGAGGAGC AAGGGTTCAA CACGATGCTA ATGTACAACA
4001 CAGGATATGG GATTGTTCCT TCATTCTGAT ACTCCTGTGG CAAAAAGAAA
4051 AACTAGATTG CAAGCTGTAA ATTACTTTTA GTTTGAGATT ATGTTAGGTT
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4151 TTGTAGTTTT GGCTAATTAA AACTATAGTA TTCTTCTTTC TCTGCATTAG
4201 T

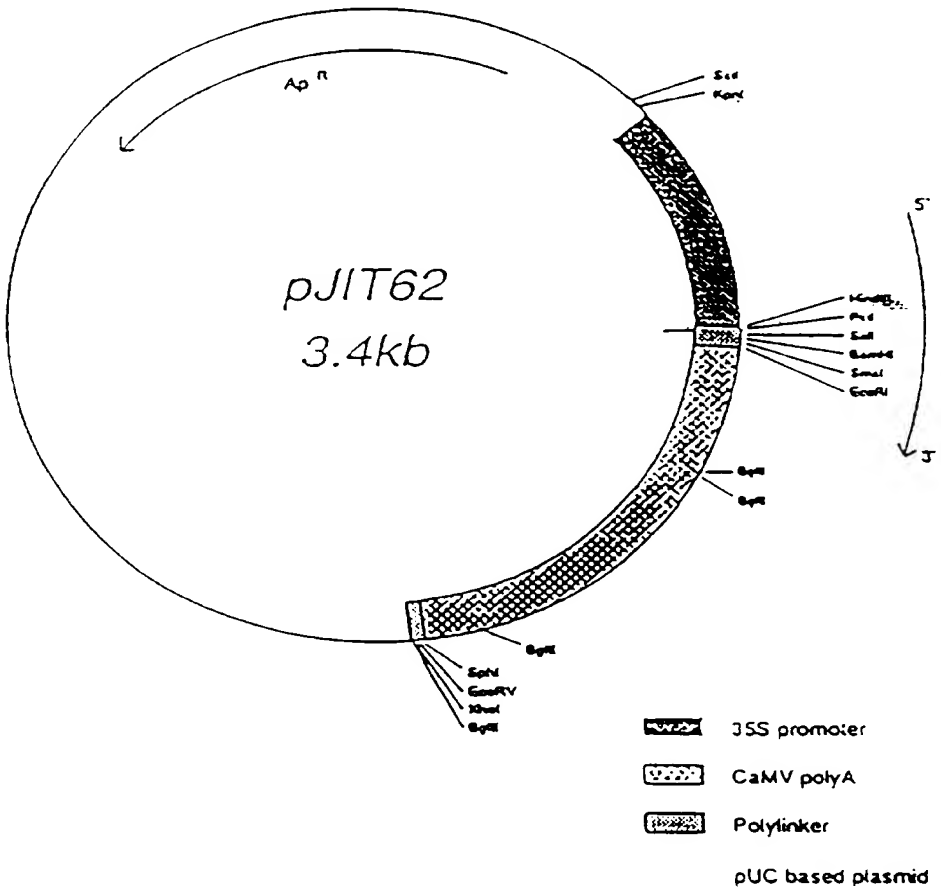
Ap R
pJIT62
3.4kb
35S promoter
CaMV polyA
Polylinker
pUC based plasmid

FIGURE 4

Figure 5.

```
1     ATGTTCAAACAAGAGAGTAACAACATTGGTAGTGAAGAGAACAACACCGGGGCGCGAGCT
      M  F  K  Q  E  S  N  N  I  G  S  E  E  N  N  T  G  A  R  A

61    TGTGACACATGCGGGTCAACCATCTGCACCGTGTACTGCCATGCTGACTCCGCCTACTTA
      C  D  T  C  G  S  T  I  C  T  V  Y  C  H  A  D  S  A  Y  L

121   TGCAATAGCTGCGATGCTCAAGTCCACTCTGCCAATCGCGTTGCTTCCCGCCATAAAAGG
      C  N  S  C  D  A  Q  V  H  S  A  N  R  V  A  S  R  H  K  R

181   GTCAGAGTGTGCGAGTCATGTGAGCGTGCCCCTGCTGCTTTTATGTGTGAGGCAGATGAT
      V  R  V  C  E  S  C  E  R  A  P  A  A  F  M  C  E  A  D  D

241   GTGTCTCTATGCACAGCCTGTGATTCAGAGGTTCACTCCGCAAACCCTCTTGCTAGACGC
      V  S  L  C  T  A  C  D  S  E  V  H  S  A  N  P  L  A  R  R

301   CATCAGCGAGTTCCAGTTGTGCCGATAACTGGAAACTCTTGCAGCTCCTTGGCCACCGCT
      H  Q  R  V  P  V  V  P  I  T  G  N  S  C  S  S  L  A  T  A

361   AACCACACAACAGTGACCGAGCCAGAGAAGAGAGTGGTGTTAGTTCAAGAGGATGCCAAA
      N  H  T  T  V  T  E  P  E  K  R  V  V  L  V  Q  E  D  A  K

421   GAGACGGCTTCATGGTTGTTCCCTAAAAACAGTGACAATCACAACAACAACAACCAGAAC
      E  T  A  S  W  L  F  P  K  N  S  D  N  H  N  N  N  N  Q  N

481   AATGAGTTGTTGTTTAGTGATGACTATCTAGACCTTGCTGATTACAACTCGAGTATGGAC
      N  E  L  L  F  S  D  D  Y  L  D  L  A  D  Y  N  S  S  M  D

541   TACAAGTTCACTGGTCAATACAATCAACCTACTCAACATAAACAAGACTGCACCGTACCA
      Y  K  F  T  G  Q  Y  N  Q  P  T  Q  H  K  Q  D  C  T  V  P

601   GAGAAAAACTACGGTGGAGATAGAGTTGTTCCACTCCAACTTGAAGAAACAAGAGGAAAC
      E  K  N  Y  G  G  D  R  V  V  P  L  Q  L  E  E  T  R  G  N

661   TTGCACCACAAGCAACATAATATCACGTATGGCTCCTCAGGAAGTCACTACAACAACAAT
      L  H  H  K  Q  H  N  I  T  Y  G  S  S  G  S  H  Y  N  N  N

721   GGTTCCATAAACCATAACGCATACAATCCATCAATGGAAACTGACTTTGTTCCGGAGCAG
      G  S  I  N  H  N  A  Y  N  P  S  M  E  T  D  F  V  P  E  Q

781   ACAGCACCTGACAAAACAGTTTCACATCCAAAAACGCACAAAGGGAAGATAGAGAAACTA
      T  A  P  D  K  T  V  S  H  P  K  T  H  K  G  K  I  E  K  L

841   CCTGAACCTCTAATTCAGATTCTCAGTCCAATGGACAGAGAAGCTAGAGTCCTGAGATAC
      P  E  P  L  I  Q  I  L  S  P  M  D  R  E  A  R  V  L  R  Y

901   AGAGAGAAGAAGAAGAGAAGAAAGTTTGAGAAGACAATAAGGTATGCTTCAAGGAAGGCA
      R  E  K  K  K  R  R  K  F  E  K  T  I  R  Y  A  S  R  K  A

961   TATGCAGAGAGAAGACCGAGGATCAATGGACGGTTTGCAAAGATTAGTGAAACCGAAGTA
      Y  A  E  R  R  P  R  I  N  G  R  F  A  K  I  S  E  T  E  V

1021  GAGGACCAAGAGTACAACACAATGCTAATGTACTATGACACAGGATATGGCATTGTTCCT
      E  D  Q  E  Y  N  T  M  L  M  Y  Y  D  T  G  Y  G  I  V  P
```

```
1081 TCATTCTATGGCCAAAAATAA
     S  F  Y  G  Q  K  *
```

Figure 6.

```
1     ATGTTCAAACAAGAGAGTAACAACATTTGTAATAGAGAGAACAACAGAGGGGCACGAGCC
      M  F  K  Q  E  S  N  N  I  C  N  R  E  N  N  R  G  A  R  A

61    TGTGACACATGCGGGTCAACCATCTGCACCGTGTACTGCCATGCTGACTCTGCCTACTTA
      C  D  T  C  G  S  T  I  C  T  V  Y  C  H  A  D  S  A  Y  L

121   TGCAATAGCTGCGATGCTCAAGTCCACTCTGCCAATCGCGTTGCTTCCCGCCATAAACGT
      C  N  S  C  D  A  Q  V  H  S  A  N  R  V  A  S  R  H  K  R

181   GTCCGGGTCTGCGAGTCATGTGAGCGTGCCCCTGCTGCTTTTATGTGTGAGGCAGATGAT
      V  R  V  C  E  S  C  E  R  A  P  A  A  F  M  C  E  A  D  D

241   GTGTCTCTATGCACAGCCTGTGATTTAGAGGTTCACTCCGCAAACCCTCTTGCTAGACGC
      V  S  L  C  T  A  C  D  L  E  V  H  S  A  N  P  L  A  R  R

301   CATCAGCGAGTTCCAGTTGTGCCGATAATTGGAAACTCTTGCAGCTCCTTGGCCACCGCT
      H  Q  R  V  P  V  V  P  I  I  G  N  S  C  S  S  L  A  T  A

          361
AACCACACAACAGTGACCGAGCCAGAGAAGAGAGTGGTGTTAGTTCAAGAGGATGCCAAA
      N  H  T  T  V  T  E  P  E  K  R  V  V  L  V  Q  E  D  A  K

421   GAGACGGCTTCATGGTTGTTCCCTAAAAACAGTGACTATCACAACAACAACAACAACCAG
      E  T  A  S  W  L  F  P  K  N  S  D  Y  H  N  N  N  N  N  Q

481   AACAATGAGTTGTTGTTTAGTGATGACTACCTAGACCTTGCTGATTACAACTCCAGTATG
      N  N  E  L  L  F  S  D  D  Y  L  D  L  A  D  Y  N  S  S  M

541   GACTACAAGTTCACCAGTCAATACAATCAACCTCGACATAAACAAGACTGCATCGTACCA
      D  Y  K  F  T  S  Q  Y  N  Q  P  R  H  K  Q  D  C  I  V  P

601   GAGAAAAACTACAGTGGAGATAGAGTTGTTCCGCTCCAACTTGAAGAAACAAGAGGAAAC
      E  K  N  Y  S  G  D  R  V  V  P  L  Q  L  E  E  T  R  G  N

661   TTGCGGAACAAGCAACAGAATATCACATATGGCTCCTCAGGAAGCCAATACAACAACAAC
      L  R  N  K  Q  Q  N  I  T  Y  G  S  S  G  S  Q  Y  N  N  N

721   GGTTCCATTAACCATAACGCATACAATCCATCAATGGAAACTGACTTTGTGCCGGAGCAG
      G  S  I  N  H  N  A  Y  N  P  S  M  E  T  D  F  V  P  E  Q

781   ACAGCACCTGACACAACAGTTTCACATCCAAAAACGCACAAAGGGAAGACAGCACAACTA
      T  A  P  D  T  T  V  S  H  P  K  T  H  K  G  K  T  A  Q  L

841   CCTGAACCTCTAATTCAGATTCTCAGTCCAATGGACAGAGAAGCTAGAGTCCTGAGATAC
      P  E  P  L  I  Q  I  L  S  P  M  D  R  E  A  R  V  L  R  Y

901   AGAGAGAAGAAGAAGAGAAGAAAGTTTGAGAAGACAATAAGGTATGCTTCAAGGAAGGCA
      R  E  K  K  K  R  R  K  F  E  K  T  I  R  Y  A  S  R  K  A

961   TATGCAGAGAGAAGACCGAGGATAAATGGACGGTTTGCAAAGATGAGTGAAACCGAAGTA
      Y  A  E  R  R  P  R  I  N  G  R  F  A  K  M  S  E  T  E  V
```

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1021 GAGGACCAAGAGTACAACACAATGCTAATGTACTGCGACACAGGATATGGCATTGTTCCT
     E  D  Q  E  Y  N  T  M  L  M  Y  C  D  T  G  Y  G  I  V  P

1081 TCATTCTATGGCCAAAAATAA
     S  F  Y  G  Q  K  *
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 95/02561

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/29 C12N15/82 A01H5/00 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOLECULAR AND GENERAL GENETICS, vol. 239, 1993 BERLIN DE, pages 145-157, PUTTERILL, J., ET AL. 'Chromosome walking with YAC clones in Arabidopsis: isolation of 1700 kb of contiguous DNA on chromosome 5, including a 300kb region containing the flowering-time gene CO' cited in the application see the whole document --- -/--	1,2,6,7, 20,21, 23-26

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
14 March 1996	27.03.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Maddox, A

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C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE PLANT JOURNAL, vol. 5, no. 2, February 1994 pages 261-272, WESTER, L., ET AL. 'Transgenic complementation of the hy3 phytochrome B mutation and response to PHYB gene copy number in Arabidopsis' see the whole document	11,12, 15,19-26
X	PLANT MOLECULAR BIOLOGY, vol. 26, October 1994 pages 657-665, CHUNG, Y-Y., ET AL. 'Early flowering and reduced apical dominance result from ectopic expression of a rice MADS box gene' see the whole document	11,12, 15,19-26
P,X	CELL, vol. 80, 24 March 1995 pages 847-857, PUTTERILL, J., ET AL. 'The CONSTANS gene of Arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors' cited in the application see the whole document	1-3, 6-12,15, 19-26
A	PLANT MOLECULAR BIOLOGY, vol. 25, June 1994 pages 335-337, AN, G., ET AL. 'Regulatory genes controlling flowering time or floral organ development' see page 335, left column, paragraph 1	16,28-32
A	THE PLANT CELL, vol. 6, no. 1, January 1994 pages 75-83, LEE, I., ET AL. 'Isolation of LUMINIDEPENDENS: a gene involved in the control of flowering time in Arabidopsis' cited in the application see the whole document	1-32
A	THE PLANT CELL, vol. 6, no. 1, January 1994 pages 1-3, CHASAN, R., ET AL. 'A time to flower' see the whole document	1-32

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Section Ch, Week 9243 Derwent Publications Ltd., London, GB; Class C06, AN 92-354683 & JP,A,04 258 292 (JAPAN TOBACCO INC) , 14 September 1992 see abstract ---	1-32
A	EMBL SEQUENCE DATABASE REL.41, 24-SEP-1994, ACCESSION NO. Z37717, MORRIS, P.C., ET AL. 'A. thaliana transcribed sequence; clone YBY029; 5' end' see the whole document -----	1